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SPECIFICITY AND PROPERTIES

OF

ANTI - HUMAN MONOCYTE SERUM

BY

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Thesis submitted to the University of Edinburgh towards
the Degree of Honours B.Sc. (Med. Sci.) in the Department
of Pathology.

Session 1974/75



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S U M M A R Y

Human leukaemic monocytes have been used to raise antisera in the rabbit and the guinea-pig with activity against a wide range of human cells.

Examination of the properties of guinea-pig anti-monocyte serum (GPAMS) in cytotoxicity and immunofluorescence tests, and in receptor studies, revealed activity against surface and cytoplasmic antigens.

Adsorption with human thymus and purified B lymphocytes resulted in the refinement of GPAMS to a restricted specificity in immunofluorescence tests for a small number of large mononuclear leucocytes. Adsorbed GPAMS was active against human macrophages.

Thus it is possible to raise a hetero-antiserum with specificity for human cells of the mononuclear phagocytic class.

GENERAL INTRODUCTION

Aim

This study investigates the properties of heterologous antiserum raised against human monocytes, determines the specificity of the antibodies produced, and observes some of their effects on the living cell.

History

The notion that antiserum could be raised by the injection of whole cells into animals is not a new one. Around the turn of the century, Eli Metchnikoff ⁽¹⁾ wrote 'J'ai émis l'idée qu'on pouvait obtenir des sérums contre toutes sortes d'éléments cellulaires', and put his idea to the test by inoculating guinea-pigs with rat spleen cells. He obtained an antiserum which distinguished between rat and mouse cells in its cytotoxic and agglutinating effects, but which was not specific for any single type of cell.

Karl Landsteiner, independently thinking along similar lines at this time, succeeded in raising antiserum against sperm.

The lead of these pioneers was followed in numerous studies during the first half of this century, interest being centred chiefly on leucocytes and cells of lymphoid tissue, because of their involvement in inflammatory and immune processes. Numerous experimenters confirmed Metchnikoff's findings, producing antisera which showed species specificity, but little or no cell-type specificity. The principal impediment to progress towards more discriminating antisera was the technical difficulty of obtaining /

obtaining a pure suspension of a single class of cells for inoculation.

In 1956 the study of heterologous antisera received fresh impetus from the discovery, by Inderbitzin, ⁽²⁾ of the immunosuppressive potency of anti-lymphocyte serum. This observation sparked off an enormous amount of research into anti-lymphocyte serum, leading to confirmation of its immunosuppressive effect in man, and the production of sera of some specificity for human lymphocytes. Anti-lymphocyte serum has found its way into therapeutics in the management of patients receiving allografts, and in the treatment of some autoimmune disorders.

Heterologous antisera against other classes of cell have been used as tools in experimental pathology. Anti-neutrophil serum, ^(3, 4) as an agent capable of effecting a selective depletion of neutrophil polymorphs, has been applied to the study of the Arthus reaction, and anti-platelet serum has been used to demonstrate the antigenic identity of platelets and megakaryocytes.

In recent years, the macrophage has received attention as a target for the production of heteroantibodies. Using animal peritoneal exudate cells, several workers have raised antisera with anti-macrophage activity, and a limited degree of specificity for this cell. These antisera have been shown to affect the in vitro expression of aspects of the macrophage's character such as phagocytosis, pinocytosis, the formation of secondary lysosomes, the expression of surface receptors, and adhesion to glass. In the presence of complement, antibodies have been shown to be cytotoxic to the cell.

A number of in vivo studies of anti-macrophage serum suggest that it has little effect on the afferent arm of the humoral immune response; Despont and Cruchaud ⁽⁵⁾ observed a reduced uptake of sheep erythrocytes by peritoneal macrophages in mice treated with anti-macrophage serum, but could demonstrate no reduction in the antibody response to sheep erythrocytes. On the other hand, antimacrophage serum has been shown to reduce the resistance to encephalomyocarditis (EMC) and yellow fever viruses in mice, ⁽⁶⁾ and to suppress the formation of hypersensitivity granulomas induced by Schistosoma mansoni eggs ⁽⁷⁾. Thus it would appear to inhibit some processes of cellular immunity.

The Present Study

a) Reasons for interest in anti-human-monocyte serum

The animal studies suggested the feasibility of the production of hetero-antiserum against the human monocyte-macrophage family of cells, and the undertaking of such a project was deemed worthwhile for several reasons. A labelled antibody specific for the macrophage would provide a criterion for the demarcation of the monocytic subclass of mononuclear cells in human peripheral blood, adding some contrast to the shades of grey which lie between monocyte and lymphocyte. A marking tag for the macrophage could prove invaluable in probing the background of some cells of disputed origin, such as the Reed-Sternberg cell of Hodgkin's disease. Finally, an antiserum raised against macrophages might contain antibodies directed against those components of the cell membrane which /

which are responsible for its considerable powers of discrimination. Antibodies to macrophage surface receptors could provide the means for a new approach to the understanding of their location, distribution and turnover.

b) Source of antigen

Despite the theoretical possibility of raising an anti-human-macrophage serum, and the obvious potential value of such an antiserum as a research tool, no description of an attempt to produce an antiserum using human material had appeared in the literature when the present work was commenced. This fact is most likely due to the difficulty of obtaining suitable material for immunisation. Peritoneal washouts from cadavers might provide a sufficient quantity of macrophages for inoculation, but the viability of cell preparations from this source is generally low. Neutrophil polymorphs could be removed by density centrifugation, but as yet no wholly satisfactory method exists for the separation of macrophages from lymphocytes. The monocyte is an alternative source of antigen, and in this project, the cells for immunisation were derived from the peripheral blood of a patient suffering from an acute monocytic leukaemia of the Schilling type, in which almost all of the circulating mononuclear cells were monocytes, eliminating the necessity of removing lymphocytes.

c) Technical approach

A /

c) Technical approach

A number of techniques can be employed to detect reactions of immune serum with cell-borne antigens. Agglutination, complement fixation, and cytotoxicity are sensitive indicators of antibody-binding to the cell. Titres obtained with dilutions of antiserum by these methods provide a basis for the comparison of the activity of serum before and after adsorption with antigens.

In the present work, the effects of anti-monocyte serum on the cell membrane has been examined by receptor studies and time-lapse cinematography, as a supplement to more traditional lines of inquiry by agglutination, cytotoxicity, and immunofluorescence techniques.

i) Cytotoxicity tests

Cytotoxicity tests have been widely used in the study of hetero-antisera to demonstrate the presence of antibodies directed against cell membrane components. Work on anti-lymphocyte serum has shown that the Ig G and Ig M fractions are equally endowed with cytotoxic antibodies, which activate complement on binding to surface antigens, causing cell lysis.

Cytotoxicity is a more direct measurement of the same fundamental reaction as that detected by complement fixation assays, and it avoids the problem of autoagglutination, a known tendency of lymphoid cells and leucocytes, which can be a source of inaccuracy in agglutination tests.

If /

If cytotoxic indices are to be used to compare the activity of an antiserum against a given cell species before and after adsorption or fractionation, it is necessary to inactivate the complement present in the antiserum, and substitute complement of known potency from fresh normal serum.

11) Immunofluorescence

Cytotoxicity tests have the disadvantage of requiring a pure cell suspension if the measurement of activity against a certain cell type is required. Immunofluorescence, in contrast, is ideally suited to the demonstration of differential antibody activity against various cell classes in a mixed population, such as that of peripheral blood.

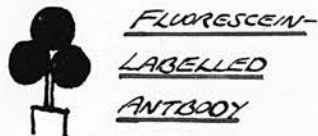
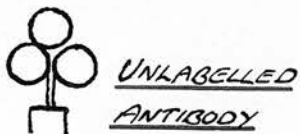
Fluorochromes can be detected in much lower-concentrations than conventional dyes; coupled to antibody, they allow the location of its binding site cytologically as well as histologically. Schroit and Gallily ⁽⁸⁾ have used immunofluorescence to show changes in the distribution of membrane-bound antimacrophage antibodies on the surface of living mouse cells with increasing periods of incubation. Immunoglobulins can gain access to intracellular structures in fixed cell preparations, and so antibodies directed against subcellular components, which would go undetected in cytotoxicity tests, can be revealed by immunofluorescence.

The indirect sandwich technique is about ten times as sensitive as /

as the direct technique, and avoids the inevitable loss of activity from the test antiserum in the process of purification and conjugation to fluorochrome. This high degree of sensitivity is attained at the price of a greater tendency for non-specific staining, the chief hazard in immunofluorescent studies, and rigorous control of tests is essential. Nairn (9) recommends the failure to stain parallel preparations treated with a middle layer of non-immune serum as a test of specificity. (Fig 1)

The determination of the identity of individual fluorescent cells in a heterogeneous population poses a problem. Phase contrast illumination of the cells under observation can sometimes provide sufficient morphological detail, but staining is required for more precise definition. As conventional dyes abolish all fluorescence, it is necessary to photograph the fluorescent cells under ultraviolet illumination, note their position on the microscope slide, stain them conventionally, and then relocate and rephotograph them. Taylor (10) has used this method successfully in a study of the human cells reacting with anti-lymphocyte serum, although he reported that cell morphology suffered during the protracted washing and staining procedures.

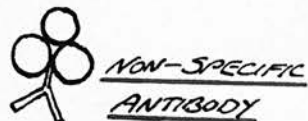
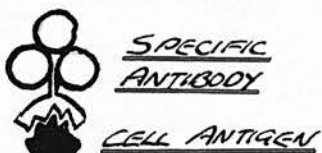
Although not generally utilised for this purpose, the fluorescent antibody technique should be applicable to the titration of antiserum. By reacting buffy coat smears with increasing dilutions of anti-monocyte serum, and noting the point of disappearance /



STEP 1

TEST

CONTROL



STEP 2

FLUORESCENCE

NO FLUORESCENCE

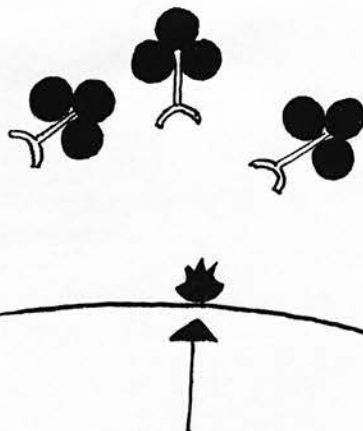
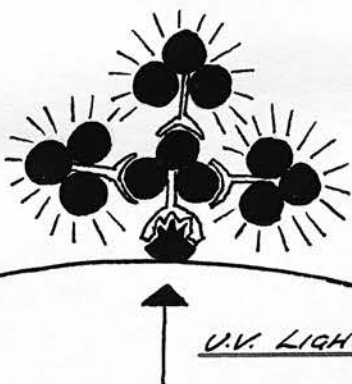


FIG. 1.
INDIRECT FLUORESCENT ANTIBODY
TECHNIQUE

disappearance of specific fluorescence of each category of cell, anti-lymphocyte, anti-polymorph, anti-monocyte, and anti-platelet activity might be titrated simultaneously.

iii) Serum fractionation

The reactions of an antiserum in immunological assays can be attributed with greater certainty to the antibodies in it if the non-immunological molecules have been removed, and many workers use purified globulin fractions in the testing of antisera. For example, whole serum can contain anticomplementary elements, causing false negative results in cytotoxicity tests. In this project, it was intended to compare the results given by whole serum and separated fractions in cytotoxicity and immunofluorescence tests before deciding which to use in the main body of work. Fractionation procedures always involve the loss of some immunoglobulin, and this is an important consideration when working with small volumes of valuable antiserum.

The two fractionation methods to be used, gel filtration and salt precipitation, have found important routine application in the preparation of purified immunoglobulins for clinical use. Gel filtration depends on the differential retardation of proteins of different sizes in their progress through a column of porous starchy beads, and salt fractionation on the different solubilities displayed by different proteins in solutions of mineral salts.

iv) Rosette inhibition

Immunologists /

iv) Rosette Inhibition

Immunologists have recently discovered cell surface receptors for the Fc portion of the Ig G molecule, and for the third component of complement. Both of these receptors are carried by macrophages, monocytes, and polymorphs, mediating the attachment of particles which have become immunologically coated, thus facilitating their phagocytosis. The phenomenon of the opsonisation of particles by antiserum has long been recognised. Human B lymphocytes also bear receptors for complement, and some bear receptors for Ig G. (11) Human T lymphocytes have a receptor for the sheep erythrocyte (SRBC) which proves useful in their identification.

The presence of these cell surface receptors can be made manifest by the addition of appropriately-coated particles, which form rosettes around receptor-bearing cells. SRBC used uncoated, Ig G-coated or complement-coated, can detect each of the three types of receptor.

The binding of large immunoglobulin molecules to the cell membrane might be expected to interfere with interactions between receptors and sensitised erythrocytes, thus resulting in inhibition of rosetting. This phenomenon can be used as a sensitive means for the detection of antibody binding to the cell surface. Bach and Antoine (12) observed that spontaneous rosetting of SRBC by mouse spleen cells was inhibited by anti-lymphocyte serum, even at high dilutions which were not cytotoxic /

cytotoxic. This observation has been extended to include human systems, and rosette inhibition has been proposed as a means of assessing the immunosuppressive potency of anti-lymphocyte serum.

Huber et al (13) have shown Ig G and complement receptors on human monocytes to be blocked by anti-lymphocyte serum.

The ability of anti-monocyte serum to inhibit rosetting by human cells was tested in this project, and the changes in its inhibitory characteristics after adsorption with different cell types were observed.

v) Adsorption

The accumulated evidence of work on heterologous antisera indicates that cells, in the process of differentiation, acquire antigens which distinguish them from other categories of cells in the same animal, and that these differentiation antigens vary from species to species. In addition, there are antigens common to cells of one animal species which can be recognised by the immune system of another species. Thus the inoculation of human cells into animals generally provokes the production of species-specific, and cell-type specific antibodies.

The purification of an antiserum containing a diversity of antibodies can be effected by adsorption; in the present case, antibodies to shared antigens were removed by adsorption onto other human cells, leaving free monocyte-specific antibodies.

It /

It was anticipated that the anti-monocyte serum would contain anti-lymphocyte activity, as anti-lymphocyte serum is known to have anti-monocyte activity. Adsorption with human thymocytes and chronic lymphatic leukaemia (CLL) cells was proposed as a means of removing the cross-reacting antibodies, since suspensions containing lymphocytes with very few monocytes or macrophages can be made from each of these sources. Thymocytes have many of the characteristics of mature T lymphocytes, and CLL cells have been shown by Ross et al ⁽¹⁴⁾ to be of B cell origin. Sequential adsorption with each of these cell types might be expected to remove all anti-lymphocyte activity.

PART 1

R A B B I T - A N T I - H U M A N - M O N O C Y T E S E R U M

: P R E L I M I N A R Y E X P E R I M E N T S

Before attempting to prepare and examine an antiserum, it was necessary to gain some practical experience of the techniques to be used. Some antiserum had previously been raised in rabbits against the leukaemic monocytes in the laboratory, and this was used in the preliminary experiments.

The aims of this part of the project were:

- (1) to develop a standard procedure for the fluorescent staining of fixed cell preparations.
- (2) to test the anti-monocyte serum for immune activity by this method, to determine the range of its activity, and to observe the effect of adsorption with erythrocytes.
- (3) to develop a cytotoxicity assay method, and to discover the cytotoxic potency of the antiserum.
- (4) to evaluate the use of whole antiserum and purified fractions of antiserum in immunofluorescence and cytotoxicity tests.

M A T E R I A L S A N D M E T H O D S

Leukaemic monocytes

The leukaemic monocytes used throughout this project, for immunisation and for testing of the antiserum, were harvested from the peripheral blood of a woman suffering from acute monocytic leukaemia of the Schilling type.

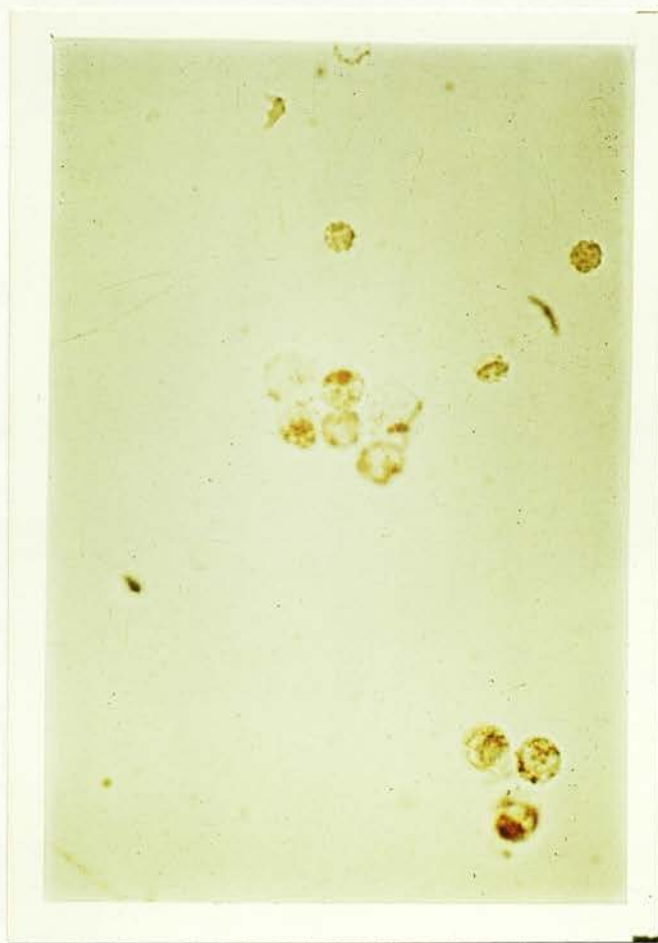
The mononuclear cells recovered by density centrifugation had the following characteristics :

Receptors:	Ig G	47%
	Complement	72%
	SRBC	1%
Surface immunoglobulin:		2%
Neutral red ingestion:		90%

90% of the cells phagocytosed Ig G-coated erythrocytes after 24 hours' culture.

The cells were suspended in 50% dimethylsulphoxide (DMSO) in heat-inactivated human serum at a concentration of 150×10^6 /ml., made into 1 ml. aliquots, and stored at -196°C in liquid nitrogen. When required for use, the cells were thawed quickly by immersion in warm water, and mixed with a large volume of a 50% solution of heat inactivated human serum in Hank's medium. Thawed monocytes showed a viability of over 90%, and retained all of their original characteristics. (Plate 1).

PLATE 1



Leukaemic monocytes showing strongly positive
ingestion of neutral red.

x 400

Immunisation

The antiserum was raised according to the following schedule :

6 outbred rabbits received 6 weekly injections of 20×10^6 leukaemic monocytes in Freund's adjuvant. The third injection was intraperitoneal, and the others were intramuscular.

The animals were bled out 10 days after the final injection.

Indirect fluorescent antibody staining

The method used was a modification of that described by Nairn.⁽⁹⁾ Air-dried smears of cells on microscope slides were fixed in 90% ethanol for 1 minute and stored at 4°C until used. Cell preparations were washed for 15 minutes in phosphate-buffered saline (PBS) (pH 8). Excessive saline was removed, and taking care not to allow drying of the preparations, dilutions of test or control rabbit serum were spotted onto the slides.

The cells were incubated at 37°C for 30 minutes in a moist chamber, rinsed, and washed 3 times for 15 minutes in PBS. A 1 in 20 dilution of fluorescein isothiocyanate-conjugated goat-anti-rabbit-Ig G (GAR-FITC) was then spotted onto the cells, again without allowing them to dry. After a further incubation at 37°C for 30 minutes, the slides were washed 3 times in PBS, and the preparation sealed under a coverslip with a drop of glycerol-buffered saline. The cells were examined under a Leitz Ortholux microscope with an HB0200 mercury vapour lamp. BG38 and BG12 excitation filters, and a K510 suppression filter.

Cytotoxicity test

The macromethod initially used was a modification of that of James.⁽¹⁵⁾ Cells were suspended in Hank's medium at a concentration of 2×10^6 /ml. Fresh human serum, diluted 1 in 4 in Hank's, was the source of complement.

Doubling dilutions of heat-inactivated test antiserum and control non-immune serum were made in 0.2 ml. volumes of Hank's in Wassermann tubes. 0.2 mls. of cell suspension and 0.2 mls. of complement were added to each dilution of serum.

Additional controls were :

- (1) cells + antiserum alone
- (2) cells + complement alone
- (3) cells + medium alone

The tubes were incubated at 37°C for 90 minutes. 0.2 mls. of freshly filtered 2% Trypan Blue was added to each tube, mixed by inversion, and held at room temperature for 10 minutes. The tubes were centrifuged at $300 \times G$ for 5 minutes, then immersed in ice water, and the supernatants discarded. The cells were resuspended in the remaining small volume of fluid, and 100 from each tube were counted in a Neubauer chamber.

Microcytotoxicity test

The micromethod was modified from that devised by Terasaki.⁽¹⁶⁾ Cells were suspended at a concentration of 1×10^6 /ml. in the serum source of complement, which was diluted according to its haemolytic potency to give 5 Minimum haemolytic doses (MHD). $2 \mu\text{l}$ volumes of cell suspension were /

were dispensed by means of a micropipette into the wells of a microtray, under a covering layer of liquid paraffin.

Doubling dilutions of heat-inactivated antiserum were made in 0.2 ml. volumes of magnesium and calcium enriched veronal buffered saline (VBS-G-Mg²⁺Ca²⁺).⁽¹⁷⁾ 2 μ l of each dilution of antiserum was added to a well, and thoroughly mixed.

Controls were : (1) cells in VBS-G-Mg²⁺Ca²⁺ alone
(2) cells in complement alone

The preparations were incubated at 37°C for 60 minutes. 0.2 ml. of freshly-filtered 1% Trypan Blue was added to each well, mixed, and held at room temperature for 15 minutes. 100 cells from each well were counted under inverted phase contrast illumination.

The cytotoxic index was defined as :

$$\frac{\text{No. of dead cells (test.)} - \text{No. of dead cells (control.)}}{\text{No. of live cells (control.)}}$$

The highest dilution of antiserum giving a cytotoxic index of 0.5 was taken as the end titre.

Complement titration

(1) Determination of the minimum haemolytic dose (MHD) of haemolysin

Rabbit anti-sheep haemolysin is routinely prepared in the laboratory according to the method of Kabat and Mayer.⁽¹⁸⁾

Doubling /

Doubling dilutions of haemolysin were made in 0.2 ml. volumes of VBS-G-Mg²⁺Ca²⁺. 0.2 mls. of 5% washed sheep erythrocytes (SRBC) in VBS-G-Mg²⁺Ca²⁺ was added to each of the dilutions of haemolysin. 0.2 mls. of reconstituted freeze-dried guinea-pig serum (Wellcome) diluted 1 in 10, was added to each well, and the preparations incubated at 37°C for 45 minutes and at room temperature for a further 45 minutes.

The highest dilution of haemolysin causing 50% haemolysis indicates the MHD50 of antibody in the SRBC system with excess complement present.

(2) Titration of complement in serum

The method of Dacie (19) was adapted for the titration of complement in the serum of normal non-immune guinea-pigs, used in cytotoxicity assays.

10 mls. of a 5% suspension of washed SRBC in VBS-G-Mg²⁺Ca²⁺ was added to 0.01 mls. of rabbit anti-sheep haemolysin diluted in 10 mls. of VBS-G-Mg²⁺Ca²⁺. After mixing by inversion, the suspension was incubated at 37°C for 30 minutes. This gave 20 mls. of 2.5% SRBC sensitised with 1.5 MHD50 of haemolysin.

Doubling dilutions of fresh guinea-pig serum were made in 0.2 ml. volumes of VBS-G-Mg²⁺Ca²⁺ in the wells of a dimple tray. To each well was added 0.2 mls. of sensitised SRBC. After /

After mixing, the preparation was incubated at 37°C for 45 minutes, and at room temperature for 45 minutes.

The highest dilution of serum causing 100% haemolysis indicated the MHD of complement in this system.

Separation of mononuclear cells from peripheral blood

Density gradient centrifugation on Ficoll Triosil was used as described by Habeshaw and Young.⁽¹⁷⁾

Haemagglutination

A 4% suspension of washed erythrocytes was made in PBS. Doubling dilutions of antiserum in 0.1 ml. volumes of PBS were made in precipitin tubes. 0.1 ml. of erythrocyte suspension was added to each dilution of antiserum, and the tubes incubated at 37°C for 45 minutes. The highest dilution of antiserum giving a broad, even carpet of agglutinated cells was taken as the end titre.

Adsorption with erythrocytes

Washed human AB erythrocytes were packed by centrifugation at 2,000 x G for 30 minutes.

5 mls. of antiserum was mixed thoroughly with 5 mls. of packed cells, and incubated at 37°C for 60 minutes.

The adsorption mixture was centrifuged at 2,000 x G for 45 minutes, the /

the serum collected, and the process repeated upon it.

Serum fractionation

(i) Gel filtration

Separation was performed on 2 columns, 25mm x 300mm, in series, packed with Sephadex G200 and G50 beads, respectively. The serum sample was drawn first through the G50 column, and then through the G200, and was eluted by Tris HCl/NaCl buffer, pH 7.9, propelled by a peristaltic pump.

The emergent protein was monitored by a LKB 8300 spectrophotometer, and collected in aliquots of 10 mls. The globulin-containing fractions were pooled and dialysed against distilled water for 36 hours at 4°C. The purified globulin was restored to the original serum volume by dialysis against polyethylene glycol.

(ii) Ammonium sulphate precipitation

γ-globulin was prepared from rabbit serum by the method of Nairn.⁽⁹⁾

Immunoelectrophoresis

The method employed was a modification of that of Ouchterlony and Nilsson.⁽²⁰⁾

2 ml. of 1.5% molten agar was poured onto a microscope slide 1 x 3ins, and allowed to solidify. The required pattern of holes and troughs was cut with a template, and rabbit serum added to the holes and stained with bromophenol blue. A constant current of 2.5 milliamps and a voltage of 100 volts was applied for approximately 2 hours. Donkey anti-rabbit-serum protein antiserum was added to the troughs, and the slides were left at 4°C overnight before inspection under indirect illumination.

R E S U L T S

Fluorescent antibody technique

Rabbit anti-monocyte serum (RAMS) agglutinated the cells of a spleen removed from a patient suffering from myeloid fibrosis. These cells were used to titrate the fluorescein-conjugated goat-anti-rabbit-Ig G antiserum (GAR-FITC), and to test the effect of adsorption of the conjugate with dried rat liver powder on the amount of non-specific fluorescence. The results are shown in Table 1.

Non-specific fluorescence was observed when GAR-FITC was used at a dilution of 1 in 10; liver adsorption effected no reduction in this non-specific fluorescence.

The most brilliant fluorescence, with no non-specific staining of cells, was observed when GAR-FITC was used diluted 1 in 20.

Range of activity of RAMS

Specific cytoplasmic fluorescence was seen in mononuclear cells, neutrophil polymorphs, and platelets of human peripheral blood buffy coat smears treated with RAMS. Erythrocytes showed no fluorescence; haemoglobin absorbs ultraviolet light and quenches fluorescence.

Two distinct patterns of fluorescence were seen, and when the cells were typed by conventional staining, it was found that the ring pattern was due to cytoplasmic fluorescence of mononuclear cells, whereas neutrophil polymorphs showed nuclear, in addition to cytoplasmic, fluorescence /

TABLE 1

Titration of fluorescein-conjugated goat and anti-rabbit Ig G (GAR-FITC)

Fluorescent staining of spleen cells by indirect technique

GAR-FITC dilution

		1 in 10	1 in 20	1 in 40	1 in 80
Unadsorbed GAR-FITC+:	RAMS 1 in 4	+++	+++	++	+
	Saline	+	0	0	0
Liver adsorbed GAR-FITC+:	RAMS 1 in 4	+++	+++	++	+
	Saline	+	0	0	0

fluorescence, which gave them a characteristic lumpy appearance.
(Plates 2 - 5)

Non-specific cytoplasmic staining of neutrophil polymorphs was observed in control preparations treated with non-immune rabbit serum. This was quite distinct from the specific staining in pattern and intensity, and in titration of RAMS, the end-point was taken as the highest dilution of antiserum showing fluorescence clearly in excess of normal rabbit serum controls.

Specific staining of both mononuclear cells and neutrophil polymorphs with RAMS titred out at a dilution of 1 in 512, but fluorescence with platelets disappeared at a dilution of 1 in 64.

Adsorption with human erythrocytes

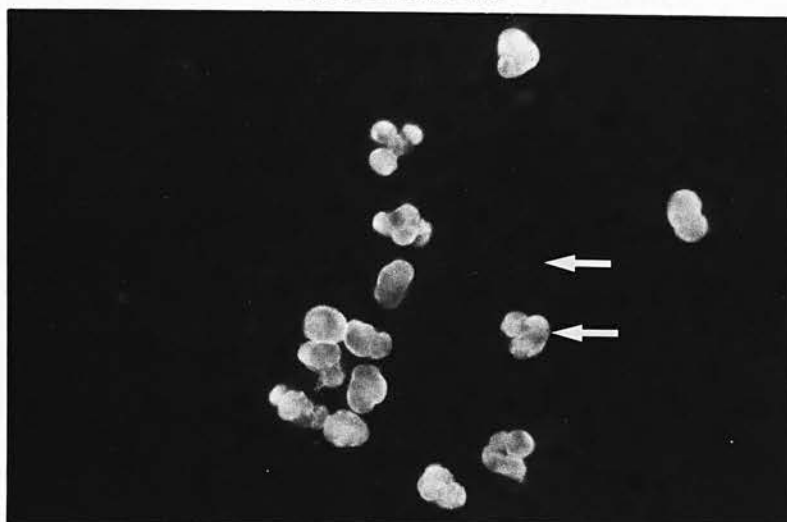
RAMS had an agglutinating titre of 1 in 128 against human group AB erythrocytes. Following adsorption, all haemagglutinating activity was abolished, but the serum retained its activity against the full range of buffy coat cells.

Cytotoxicity

Using the macromethod described in materials and methods, no cytotoxicity towards human mononuclear leucocytes was detected. The method was modified further, in an attempt to unveil any cytotoxic activity in the serum.

a) Incubation period /

PLATE 2



Erythrocyte

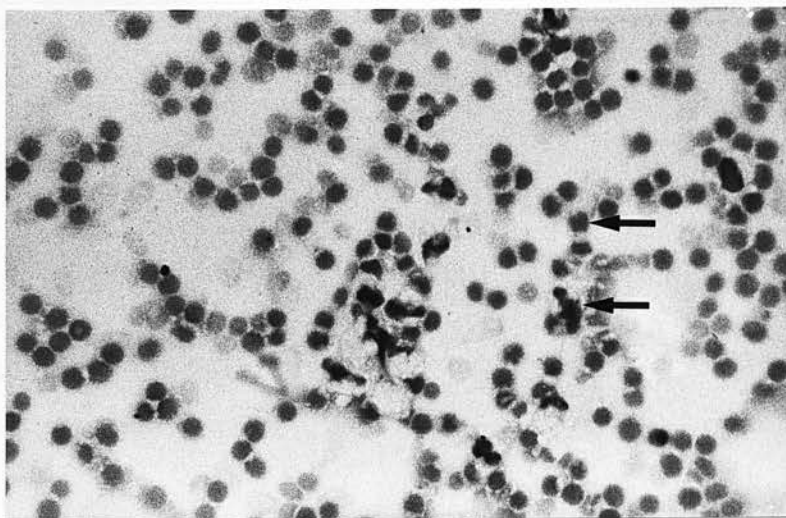
Neutrophil polymorph.

Strongly fluorescent neutrophil polymorphs,
and negative erythrocytes.

RAMS diluted 1 in 4

x 400

PLATE 3



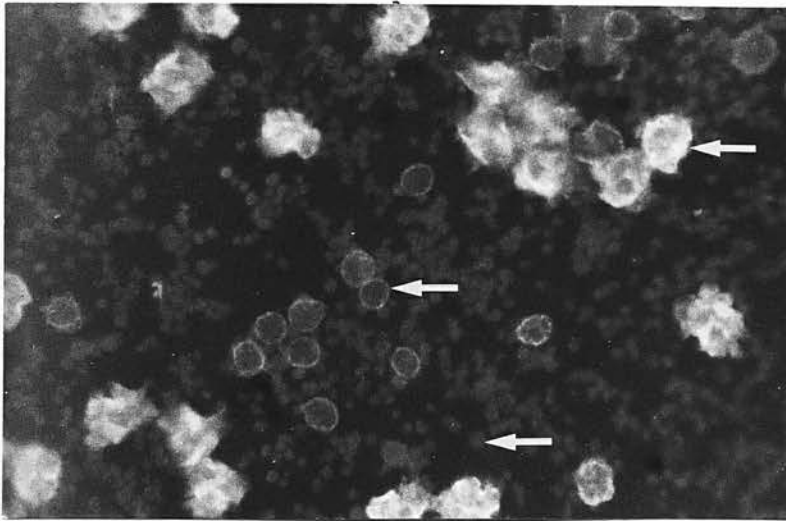
Erythrocyte

Neutrophil Polymorph.

Same field as Plate 2, Giemsa stain.

x 400

PLATE 4



Neutrophil polymorph.

Mononuclear cell.

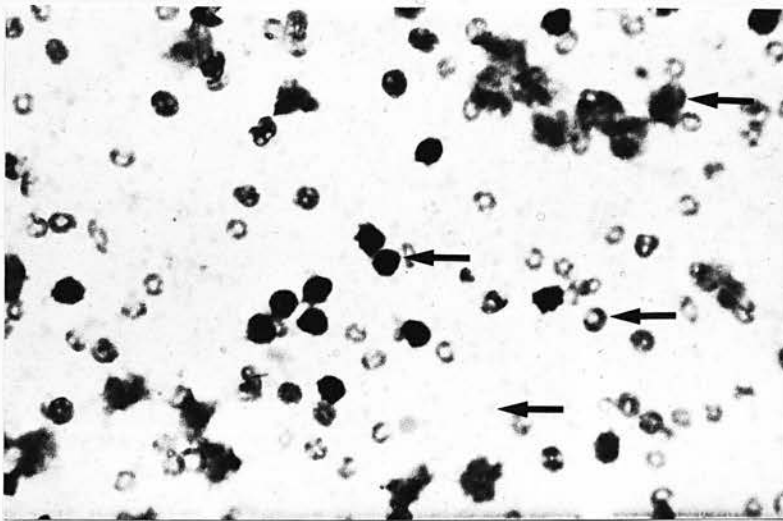
Platelet.

Buffy coat preparation showing fluorescent
neutrophil polymorphs, mononuclear cells, and
platelets.

RAMS diluted 1 in 4

x 400

PLATE 5



Neutrophil polymorph

Mononuclear cell

Erythrocyte

Platelet

Same field as Plate 4, Giemsa stain.

x 400

a) Incubation period

Lengthening of the incubation period decreased the viability of cells in test and control preparations, and failed to reveal cytotoxicity.

Prolonged incubation of cells at 37°C was shown to lead to progressive loss of viability. (Fig. 2) An incubation time of 1 hour was chosen.

b) Medium

VBS-G-Mg²⁺Ca²⁺, a medium with the optimal concentration of the divalent cations necessary for complement activity, was substituted for Hank's medium.

c) Source of complement

The consistently negative results were found to be due to the inefficacy of human serum as a source of complement in this system, despite its potency in haemolytic assays. When normal guinea-pig serum was used as the complement source, RAMS was seen to be strongly cytotoxic to human mononuclear leucocytes.

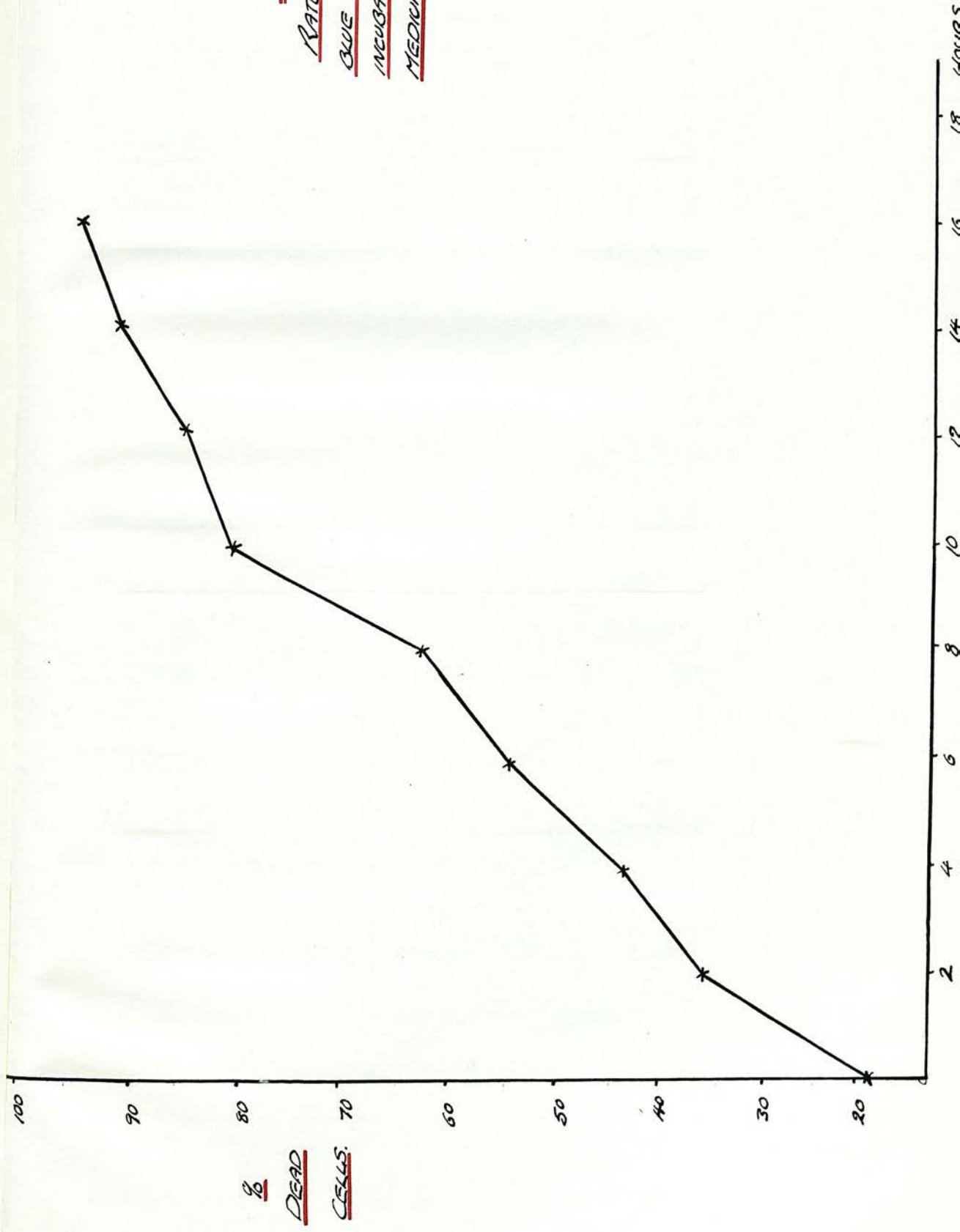
d) Micromethod

The micromethod finally adopted subjected the cells to essentially the same treatment as the macromethod, but allowed much faster counting of results, and great economy in materials.

e) Counting error

When /

FIG 2.
RATE OF UPTAKE OF TRYPA
BLUE BY LEUKAEMIC MONOCYTES
INCUBATED AT 37°C IN OUFERED
MEDIUM.



e) Counting error

When 10 counts of 100 cells from different microscopic fields in a single well of the microtray were made, a range of 11% in results was obtained.

Cytotoxic and haemagglutinating titres

In cytotoxicity tests, RAMS was observed to have a duality of activity, agglutinated viable cells being seen in addition to dead cells.

The respective titres of these components against human mononuclear leucocytes were :

Agglutination	<u>1 in 512</u>
Cytotoxicity	<u>1 in 256</u>

Purification of globulin

Gel exclusion chromatography effected the separation of RAMS into two peaks, (Fig. 3) the first eluted containing the antibody activity, and consisting predominantly of globulin. Ammonium sulphate precipitation of normal rabbit serum obtained a cleaner separation of immunoglobulin. The immunoelectrophoretic profile of whole serum and the separated fractions is shown in Fig. 4. RAMS was seen to have a strong Ig M component. Both separation procedures resulted in the loss of Ig M from the globulin fraction. A more complete separation of serum proteins could have been achieved by the use of a longer column in gel /

FIGURE 3

Spectrophotometer record of eluted protein
fractions from gel electrophoresis separation
of RAMS

VEDEN. CHART No. 6522 A Imp.

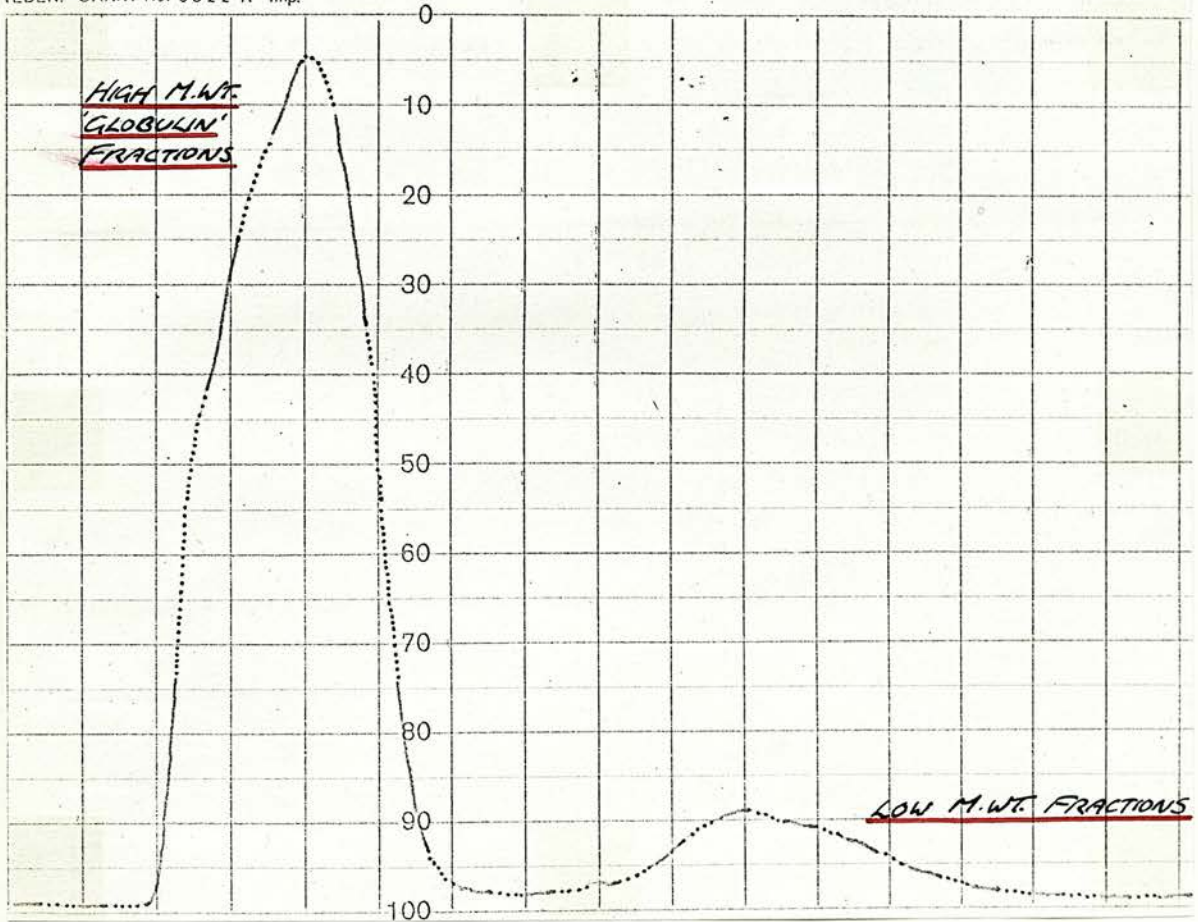
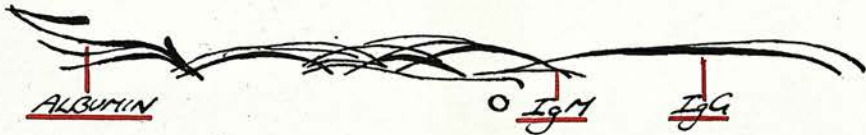


FIGURE 4

Immunoelectrophoretic profile of whole and fractionated normal rabbit serum and RAMS

1) Whole normal rabbit serum.



2) Whole RAMS.



3) Normal rabbit serum : salt precipitated
'globulin' fraction.



4) RAMS : gel exclusion 'globulin' fraction.



gel exclusion chromatography.

When the serum fractions were applied in immunofluorescence, some reduction in the nonspecific staining of neutrophil polymorphs was observed.

As whole serum had given unambiguous, positive results in immunofluorescence and cytotoxicity assays, and the separation methods used clearly required further refinement before they could be of value, it was decided to use whole serum throughout the rest of the project.

CONCLUSIONS

- (1) A suitable method for the titration of antibodies directed against cells, by the indirect fluorescent antibody technique, had been developed.
- (2) RAMS was active against human erythrocytes, platelets, neutrophil polymorphs, and mononuclear leucocytes. Anti-red cell antibodies had been removed with no reduction in the scope or intensity of anti-leucocyte activity.
- (3) A cytotoxicity assay had been successfully adapted for the detection of complement-fixing antibodies directed against the surface of human cells.
- (4) Fractionation techniques had not been employed successfully in the preparation of purified immunoglobulin, and whole serum was satisfactory for use in immunofluorescence and cytotoxicity assays.
- (5) RAMS contained a strong component of agglutinating, non-complement-fixing antibodies.

PART 2

GUINEA - PIG - ANTI - HUMAN - MONOCYTE SERUM

: SPECIFICITY AND REACTIONS

Having shown that the leukaemic monocytes had stimulated the production of an active antiserum in the rabbit, and having found immunofluorescence and cytotoxicity methods suitable for the detection of antibodies against cell components, it was possible to proceed in some confidence with the preparation and examination of another anti-monocyte serum. A different animal species, the guinea-pig, was used.

The aims of this part of the project were :

- (1) to prepare an antiserum to human monocytes in the guinea-pig.
- (2) to define the range of its activity against human material.
- (3) to observe the effect of adsorption with leukaemic monocytes, erythrocytes, thymocytes, and B lymphocytes, and hence to determine the specificity of its constituent antibodies.
- (4) to investigate its interactions with the cell surface by receptor studies.
- (5) to observe its effect on the behaviour of living human cells in a monolayer culture by time-lapse cinematography.

MATERIALS AND METHODS

Immunisation

Each of 6 outbred young female guinea-pigs was given an intraperitoneal injection of 80×10^6 leukaemic monocytes, followed by 2 further injections of 40×10^6 cells at weekly intervals, by the same route.

The serum of one animal was tested for immune activity 5 days after the second injection, and the others were bled out 7 days after the final injection. The sera were tested individually for immune activity, and then pooled and heat inactivated.

Adsorption

a) Cells

Thymocytes - were obtained from the thymuses of children, partially resected during cardiac surgery. All showed normal histology. Within 60 minutes of excision, the organ was minced and strained, and the cells suspended and washed 3 times in Hank's medium. In each case, the suspension obtained was 50% viable, and of the viable cells, about 80% formed rosettes with SRBC.

B lymphoma cells - were collected as above from the supraclavicular node resected from a patient suffering from a nodular B cell lymphoma.
The /

The suspension obtained was 75% viable, and the cells had the following characteristics :

Surface immunoglobulin	71%
Receptors : SRBC	27%
Ig G	25%
Complement	52%
Neutral red ingestion	1%

Chronic lymphatic leukaemia (CLL) cells - were prepared by density centrifugation of the blood of a patient suffering from CLL. The suspension was over 95% viable, and the cells showed these characteristics :

Surface immunoglobulin	74%
Receptors : SRBC	4%
Ig G	64%
Neutral red ingestion	2%

b) Procedure

A suspension containing 2.5×10^8 viable cells was centrifuged at 500 x G for 10 minutes, the supernatant discarded, and the tube inverted for 2 minutes to 'dry' the cells.

0.5 mls. of antiserum was added, and the cells resuspended and incubated at 37°C for 60 minutes.

The /

The mixture was then centrifuged at 2,000 x G for 15 minutes, the supernatant serum collected, and the adsorption process repeated.

Neutral red ingestion

The method of Habeshaw and Young ⁽¹⁷⁾ was used.

Surface immunoglobulin

The method used for its detection by indirect immunofluorescence was that of Habeshaw and Young. ⁽¹⁷⁾

Double diffusion

A 1% agar solution containing 0.8% NaCl was poured onto a Petri dish to a depth of 3mm and allowed to cool and congeal. One central, and 5 peripheral wells were punched in the agar. The central well was filled with human serum, and the peripheral wells with antisera. The preparation was allowed to stand at 4°C overnight before inspection under indirect lighting.

Time-lapse cinematography

Leukaemic monocytes were cultured on flying coverslips within test tubes, and introduced into sterile chambers for observation, according to the method devised by Stuart et al. ⁽²¹⁾

The /

The equipment used in time-lapse cinematography is illustrated in Fig. 5. Exposures were made at a rate of 1 per 5 seconds or 1 per second, depending on the activity of the cells under observation.

Rosette inhibition

a) Preparation of rosettes

The materials and methods used in the preparation of rosettes were those of Habeshaw and Young.⁽¹⁷⁾

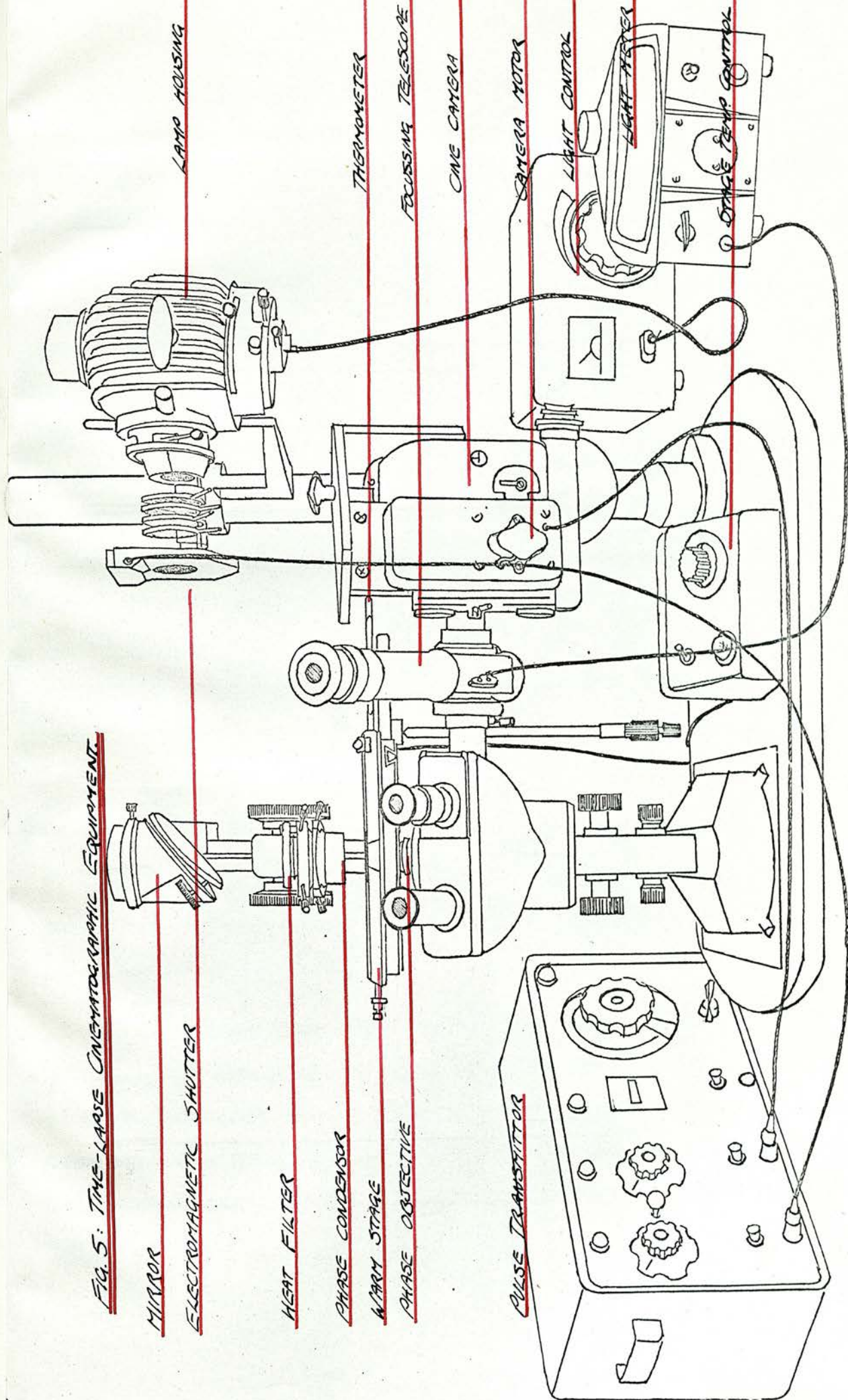
200 cells were counted. Rosetting cells were defined as those binding 3 or more erythrocytes.

The viability of the white cell population was determined by Trypan Blue exclusion.

b) Inhibition of rosettes

1×10^6 cells were incubated at 37°C for 60 minutes with heat inactivated GPAMS diluted 1 in 6 in PBS. The cells were washed 3 times in PBS, and rosettes prepared and counted as above.

FIG. 5: TIME-LARGE CINEMATOGRAPHIC EQUIPMENT.



RESULTS

Immunofluorescence

a) Range of activity

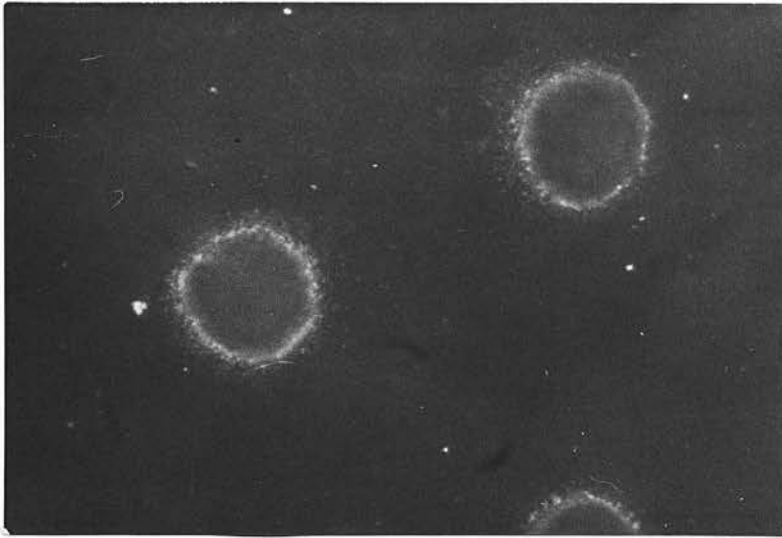
The guinea-pig anti-human-monocyte serum (GPAMS) had a wide range of activity against human cells. It caused brilliant fluorescence of the leukaemic monocytes used for inoculation. (Plate 6) These cells showed slight nonspecific fluorescence with non-immune serum in controls. (Plate 7) In addition, specific fluorescence was seen with human chronic lymphatic leukaemia (CLL) cells, normal thymocytes and peripheral blood mononuclear cells, neutrophil polymorphs, and platelets (Table 2). GPAMS activity against mononuclear leucocytes and neutrophil polymorphs in buffy coat preparations showed identical titres, but anti-platelet activity was lost at a lower dilution.

b) Adsorption

i) Leukaemic monocytes GPAMS when adsorbed with leukaemic monocytes was rendered completely inert in immunofluorescence tests with all of the above cell species.

ii) Human thymocytes Thymocyte-adsorbed GPAMS was negative with respect to thymocytes, but showed strong fluorescence with leukaemic monocytes, neutrophil polymorphs, and a small percentage of mononuclear cells from normal human peripheral blood. (Table 3) In order to determine whether this percentage corresponded numerically to that of any subclass of human mononuclear /

PLATE 6



Leukaemic monocytes showing strongly positive
ring staining.

GPANS diluted 1 in 4

x 1000

PLATE 7



Leukaemic monocytes showing weak nonspecific
staining.

Normal guinea-pig serum diluted 1 in 4

Same exposure and development times as
Plate 6

x 1000

TABLE 2

Titre of activity of GPAMS against human cells by immunofluorescence

	Leukaemic Monocytes	Thymocytes	Neutrophil Polymorphs	Platelets	Mononuclear Leucocytes	CLL Cells
GPAMS	128	128	1024	256	1024	16
Monocyte adsorbed GPAMS	0	0	0	0	0	Not Done

Figures are reciprocal of highest dilution of antiserum showing specific fluorescence

TABLE 3

Activity of adsorbed GPAMS against human cells by immunofluorescence

	Leukaemic Monocytes	Thymocytes	Neutrophil Polymorphs	Platelets	Mononuclear Leucocytes	CLL Cells
Thymocyte- adsorbed GPAMS	++	0	++	0	*++	Not Done
Thymocyte- and CLL- adsorbed GPAMS	++	0	++	0	*++	0

* approx. 12% of cells were strongly positive; the rest were negative

mononuclear leucocytes, a sample of peripheral blood was separated on a Ficoll/Triosil gradient, and the receptor, surface immunoglobulin, and phagocytic characteristics of the mononuclear suspension defined. Fixed preparations of the suspension were then treated with thymocyte-adsorbed GPAMS by immunofluorescence. The results are shown in Table 4.

Mononuclear cells which ingest neutral red are considered to be monocytes; whereas these accounted for 3% of the cells, thymocyte-adsorbed GPAMS reacted with 13%. (Plates 8 and 9) This discrepancy could be explained by the presence of monocytes which were not actively phagocytic, and the figure of 13% is close to the average number of circulating monocytes in man. However, the possibility that the adsorbed antiserum also reacted with a number of lymphocytes antigenically distinct from thymocytes had to be investigated.

iii) Thymocytes + CLL cells Adsorption with CLL cells, which were predominantly B-cell in character, was calculated to remove any anti-B-lymphocyte activity.

This doubly-adsorbed antiserum, now inactive against human thymocytes and CLL cells, caused strong fluorescence with the leukaemic monocytes (Plate 10) and delineated the same number (12%) of mononuclear leucocytes as the thymocyte-adsorbed GPAMS. (Plates 11 - 14) Of these cells, some had distinct monocyte morphology when viewed under phase contrast illumination, but others could not be identified in this way. Conventional staining of preparations which had been treated by immunofluorescence was of too poor a quality to allow discernment of the finer /

TABLE 4

Characteristics of mononuclear leucocyte preparation
treated with thymocyte-adsorbed GPAMS

Giemsa stain:

Neutrophil polymorphs	15%
Mononuclear cells	85%

Surface receptors:

SRBC	61%
Ig G	61%
Complement	55%

Phagocytosis

Neutral red ingestion	3%
-----------------------	----

Surface immunoglobulin

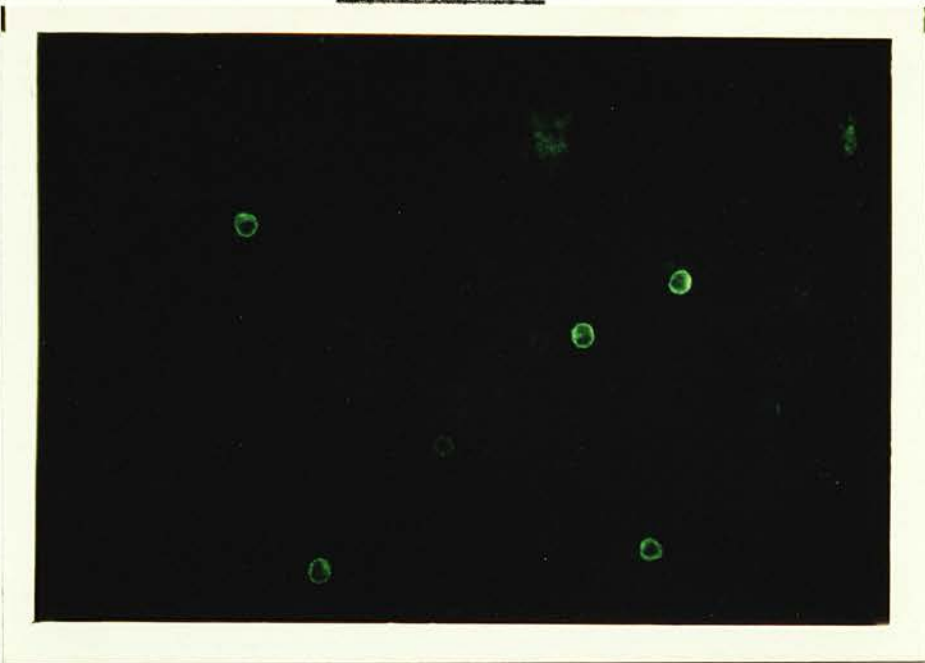
Fluorescent capping	24%
Fluorescent non-capping	2%

Fluorescence with thymocyte-adsorbed GPAMS:

13% of mononuclear cells

500 cells counted.

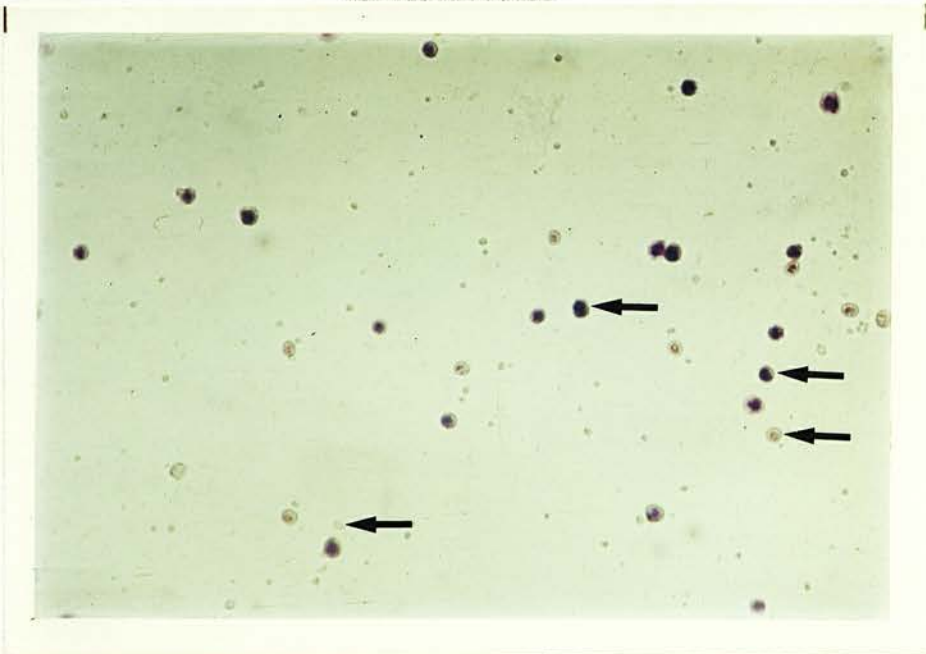
PLATE 8



Fluorescence of five cells in a human mononuclear leucocyte preparation. Thymocyte-adsorbed GPAMS diluted 1 in 2

x approx. 300

PLATE 9

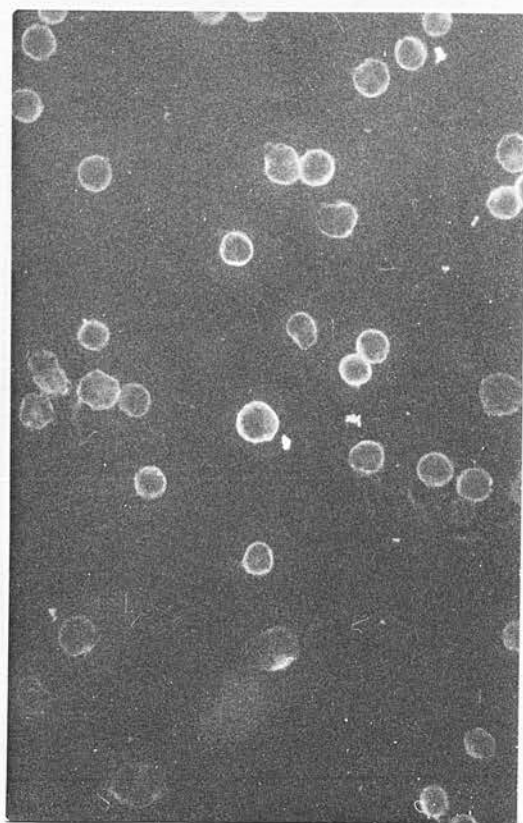


*Fluorescent
leucocyte*
*Non-Fluorescent
leucocyte*
Erythrocyte
Platelet.

Same field as Plate 8, Giemsa stain.

x 250

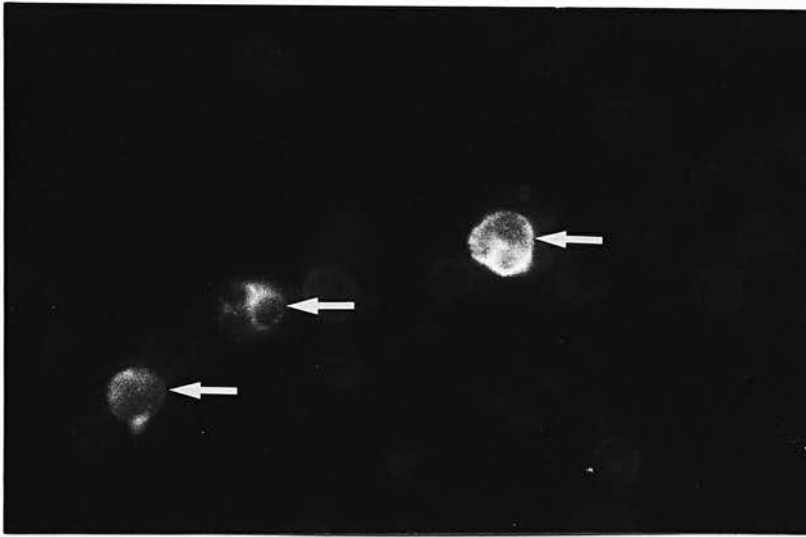
PLATE 10



Strong ring fluorescence of leukaemic monocytes.
Thymocyte and CLL-adsorbed GPAMS, diluted 1 in 2

x 250

PLATE 11



Monocyte

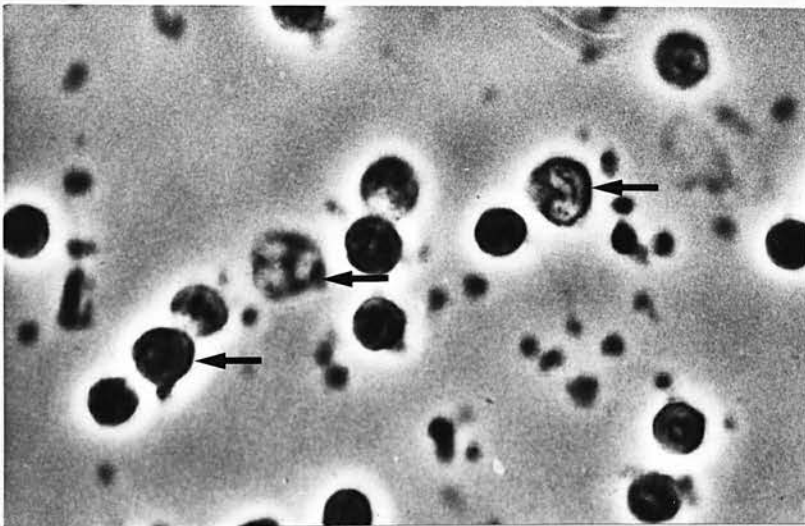
Neutrophil polymorph.

Mononuclear cell

Strong specific fluorescence in three cells in
a human leucocyte preparation. Thymocyte and
CLL-adsorbed GPAMS diluted 1 in 2

x 400

PLATE 12

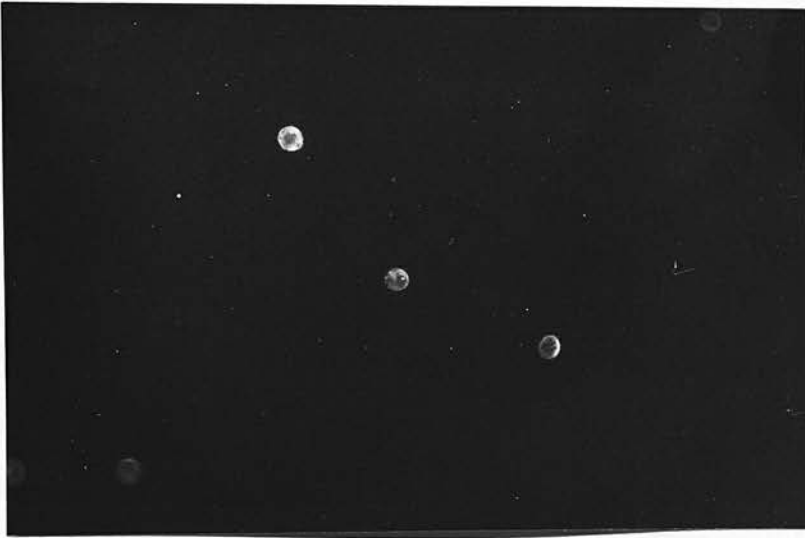


Fluorescent cells.

Same field as Plate 11, phase contrast
illumination.

x 400

PLATE 13

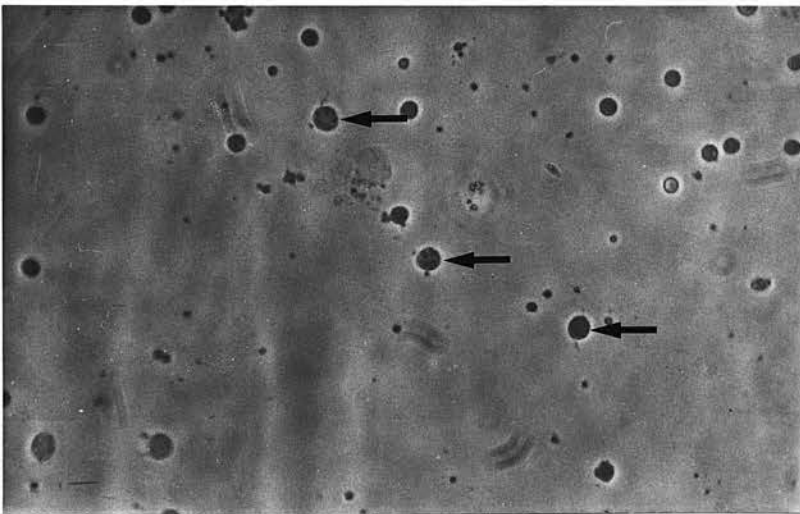


Bright specific fluorescence in three large cells
of a human mononuclear leucocyte preparation.

Thymocyte and CLL-adsorbed GPAMS diluted 1 in 2

x 250

PLATE 14



Fluorescent cells.

Same field as Plate 13, phase contrast illumination.

x 250

finer details of morphology.

Anti-neutrophil polymorph activity was still evident in the GPAMS which had been adsorbed with thymocytes and CLL cells, but anti-platelet activity disappeared after adsorption with thymocytes.

Anti-erythrocyte activity

GPAMS had a haemagglutinating titre of 1 in 256, and a haemolytic titre of 1 in 24, against human group O erythrocytes.

Anti-macrophage activity

GPAMS adsorbed with thymocytes and erythrocytes caused strong cytoplasmic fluorescence in the cells of a 24 hour culture of human leucocytes.

(Plates 15 - 20) Similar reactivity was seen against histiocytes cultured from a human spleen.

Anti-human-serum-protein activity

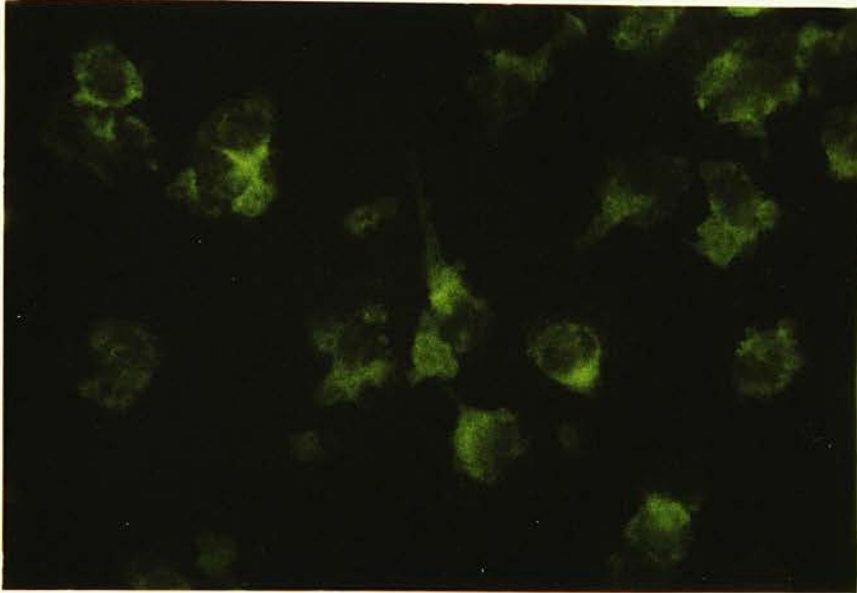
GPAMS was devoid of reactivity against human serum proteins in double diffusion precipitation tests.

Cytotoxicity

a) Range of activity

GPAMS was cytotoxic in the presence of complement to leukaemic monocytes,
human /

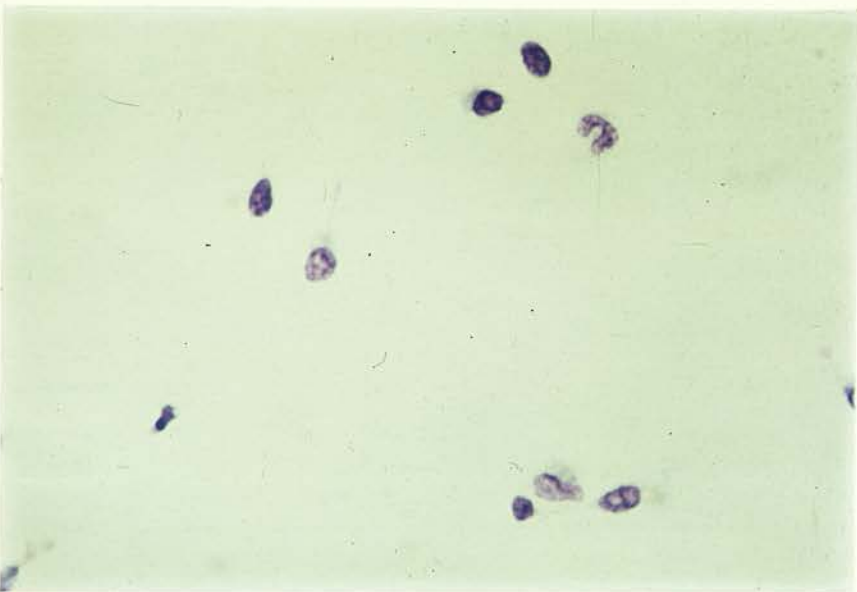
PLATE 15



Cytoplasmic fluorescence in cells of 24 hr. culture
of human peripheral blood mononuclear cells.
Thymocyte and erythrocyte-adsorbed GPAMS diluted
1 in 4

x approx. 500

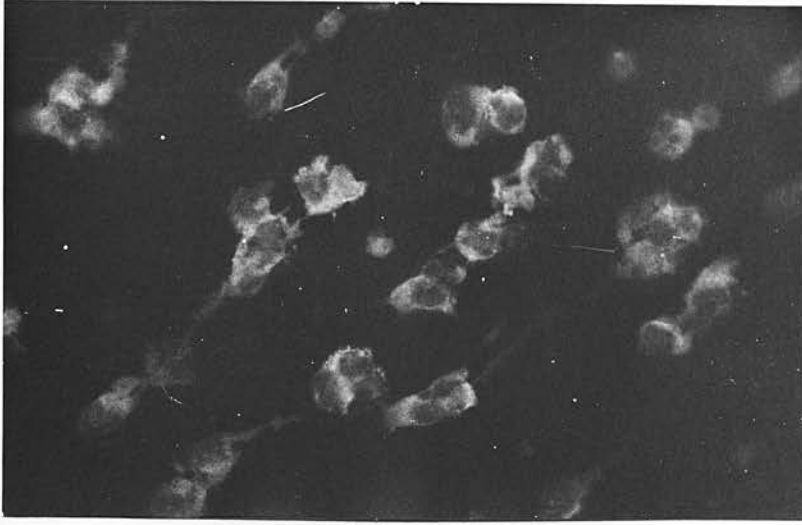
PLATE 16



Field from same culture as in Plate 15, Giemsa
stain.

x 400

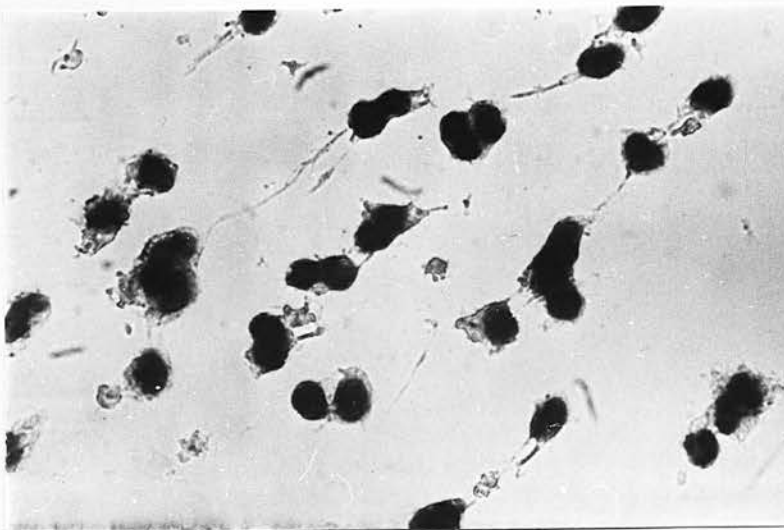
PLATE 17



Cytoplasmic fluorescence in cells of a 24 hr. culture of human peripheral blood mononuclear cells. Thymocyte and erythrocyte-adsorbed GPAMS diluted 1 in 4

x 400

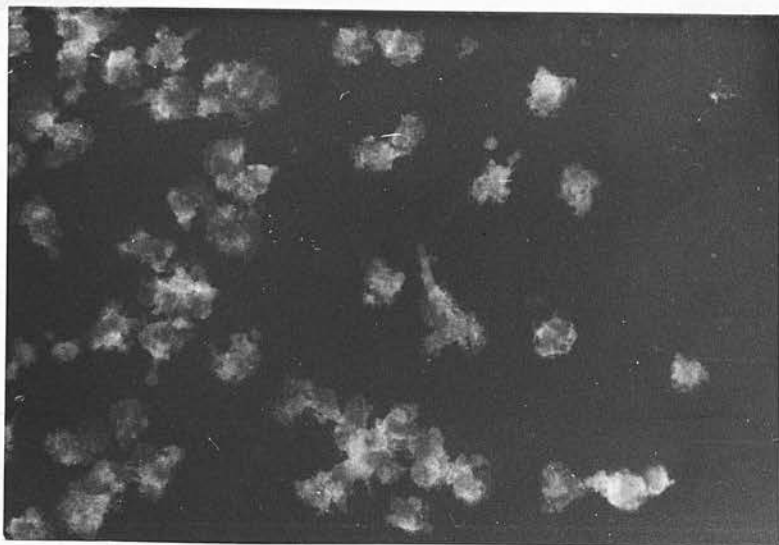
PLATE 18



Same field as Plate 17, Giemsa stain

x 400

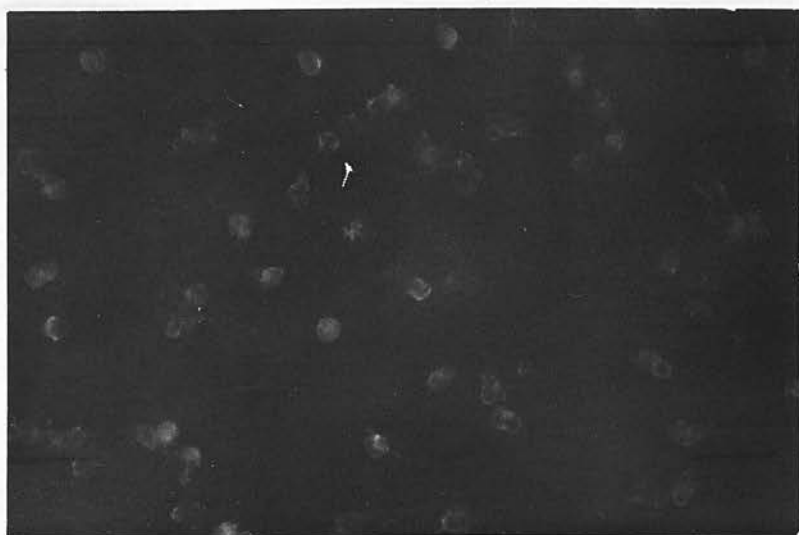
PLATE 19



Cytoplasmic fluorescence in cells of 24 hr. culture
of human peripheral blood mononuclear cells.
Thymocyte and erythrocyte-adsorbed GPAMS diluted
1 in 2

x 250

PLATE 20



Weak nuclear fluorescence in culture similar to
that of Plate 19. Non-immune guinea-pig serum,
diluted 1 in 2

x 250

human thymocytes, CLL cells, neutrophil polymorphs, and mononuclear leucocytes. (Table 5)

b) Adsorption

i) Leukaemic monocytes When GPAMS was adsorbed with leukaemic monocytes, it lost all cytotoxicity.

ii) Human thymocytes GPAMS adsorbed with thymocytes was no longer cytotoxic to either thymocytes or to leukaemic monocytes. A cytotoxic effect against human mononuclear leucocytes was noted with low dilutions of thymocyte-adsorbed GPAMS. Although the cytotoxic index did not reach 0.5, approximately 15% of mononuclear cells were killed.

iii) Thymocytes + CLL cells Double adsorption with these two species of cells removed all cytotoxicity against human mononuclear leucocytes.

Receptor studies

The activity of GPAMS against the cell surface was investigated by testing its ability to inhibit cell membrane receptors.

a) Effects of unadsorbed GPAMS on cell surface receptors

i) Leukaemic monocytes

a) Thymocyte-adsorbed: GPAMS which had been adsorbed with human thymocytes continued to cause almost total inhibition of the Ig G receptor on the leukaemic monocyte. Inhibition of the complement receptor /

TABLE 5

Cytotoxicity of GPAMS to human cells

	Leukaemic Monocytes	Thymocytes	Neutrophil Polymorphs	Mononuclear Leucocytes	CLL cells
GPAMS	128	128	32	128	128
Monocyte adsorbed GPAMS	0	0	Not Done	0	Not Done
Thymocyte adsorbed GPAMS	0	0	Not Done	* 0	Not Done

* 15% of cells were killed by low dilutions of antiserum.

Figures are reciprocal of highest dilution of antiserum giving cytotoxic index of 0.5.

receptor was decreased by adsorption. (Table 6)

b) Thymocyte- + B lymphoma cell-adsorbed: Following a double adsorption on thymocytes and lymphoma cells which were predominantly B lymphocytes, GPMS continued to show very strong inhibition of the Ig G receptor on leukaemic monocytes. The complement receptor was now virtually uninhibited.

ii) Mononuclear leucocytes In order to define the population of mononuclear leucocytes with which thymocyte-adsorbed GPMS reacted, these cells were incubated with the adsorbed antiserum in the presence of complement, and the inhibitory effect on their surface receptors observed. Whilst only 25% of the cells were killed, all three receptors were totally inhibited, (Table 7) indicating that the adsorbed antiserum had retained its surface activity against the whole spectrum of mononuclear leucocytes. This reaction was not entirely complement-dependent; thymocyte-adsorbed GPMS alone caused a lesser inhibition of the three types of receptor.

Effect of GPMS on the behaviour of living cells

The cytotoxic effect of the antiserum on leukaemic monocytes in culture was observed under phase contrast illumination, and recorded by time-lapse cinematography.

In culture, the leukaemic monocytes developed into typical macrophages; glass-adherent, motile, and highly phagocytic. (Plate 23). During the first few hours of culture, several of the cells underwent unusual changes in conformation, developing short, rounded cytoplasmic protrusions and continuously /

TABLE 6

Effect of GPAMS on receptors of leukaemic monocytes

		<u>Percentage inhibition</u>		
		Receptor :		Viability :
Nature of Antiserum:			Ig G	Complement
	GPAMS	94	91	85%
	Thymocyte- adsorbed GPAMS	97	71	85%
	Thymocyte- and B cell-adsorbed GPAMS	81	5	85%

$$\% \text{ inhibition} = \frac{\text{no. of control rosettes} - \text{no. of test rosettes}}{\text{no. of control rosettes}} \times \frac{100}{1}$$

Control: cells incubated with non-immune guinea-pig serum.

TABLE 7

Effect of thymocyte-adsorbed GPAMS on receptors of human mononuclear leucocytes

Percentage inhibition

Receptor :

Viability :

Ig G

Complement

SRBC

Thymocyte-
adsorbed
GPAMS
+ complement

100

99

96

65%

Thymocyte-
adsorbed
GPAMS
alone

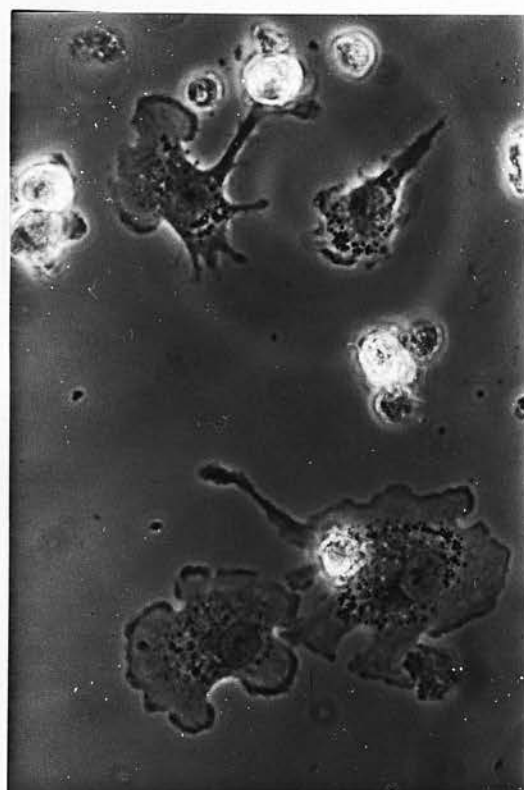
75

47

30

95%

PLATE 23



Macrophage-like morphology in 72 hr. culture
of leukaemic monocytes.

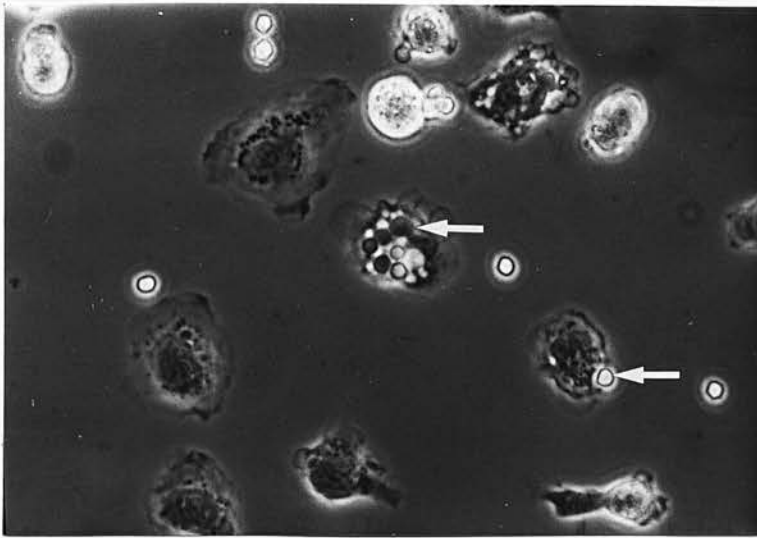
x 250

continuously altering shape over a period of minutes, before resuming a spheroidal form. (Plate 21) By 24 hours, half of the cells were adherent to the glass, and had become large and outspread, and by 72 hours almost all were typical macrophages. (Plate 22)

Addition of antiserum and complement, diluted 1 in 7, to the medium, initiated a sequence of changes in the cells. Movement was inhibited almost immediately, and within 20 minutes, blebs began to form in the cell membranes. As these blebs broke free from the cells, the medium became crowded with small spherical cytoplasmic fragments. Subcellular organelles, which appeared as phase-dense spots, were seen to enlarge and lose definition. Pseudopodia were gradually withdrawn over a period of 3 hours, during which the nuclei became increasingly ill-defined, until finally most cells had the appearance of shrunken, irregular spheres.

When GPMS and complement were added at a dilution of 1 in 2, the effect was immediate and dramatic. The large spreading cells rapidly withdrew their cytoplasmic extensions within 10 to 15 minutes, whilst the nuclei appeared to shrink. After 15 minutes, shrunken cells showed a sudden, slight swelling in profile, and then remained motionless, their cytoplasmic structures condensed, fragmented, and irregularly outlined. (Fig. 6)

PLATE 20



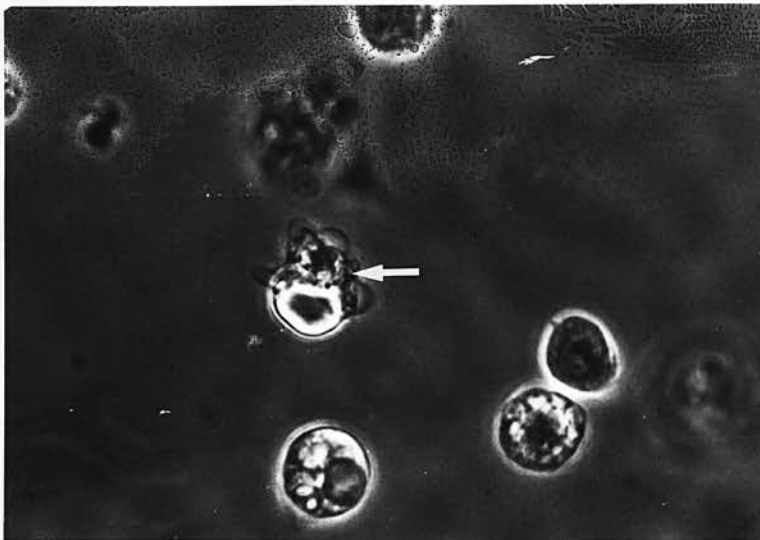
Intracellular SRBC

Adherent SRBC

Phagocytosis of opsonised SRBC in 72 hr. culture
of leukaemic monocytes.

x 250

PLATE 22



Cytoplasmic protrusions.

Cell undergoing morphological change in 12 hr.
culture of leukaemic monocytes.

x 400

FIGURE 6

Diagram of the morphological changes in a glass-adherent human monocyte during a period of 15 minutes after exposure to GPMS and complement, drawn from time-lapse cinematographic frames.

x approx. 450

C O N C L U S I O N S

- (1) An antiserum had been raised in the guinea-pig, against surface and cytoplasmic antigens of human leukaemic monocytes.
- (2) The antiserum had a wide range of activity against normal human leucocytes, thymocytes and erythrocytes, and against CLL cells.
- (3) Adsorption studies showed that a proportion of the constituent antibodies was directed against shared cell antigens, and a proportion was specific for monocytes and macrophages, neutrophil polymorphs, and possibly a small number of related mononuclear leucocytes.
- (4) Inhibition of the receptor for Ig G was a sensitive indicator of the interaction of antibodies with the surface of the monocyte.
- (5) Anti-monocyte serum had a number of effects on living cells in culture, including inhibition of movement, retraction of processes, and cytoplasmic blebbing.

DISCUSSION

A) Techniques

Immunofluorescence

It was found that immunofluorescence could be used as a sensitive technique for the titration of antibodies directed against antigens located in any part of the cell. Specific activity was detected by this method after all demonstrable cytotoxicity had been adsorbed out of the antiserum.

The technique was applied successfully to the titration of activity against individual cell types in peripheral blood buffy coat preparations, and was able to show that the anti-platelet activity of RAMS and GPAMS was lost on dilution before the anti-neutrophil polymorph and anti-mononuclear activity.

Cells which had been observed, fluorescing, under ultraviolet light, and then stained by conventional dyes, could not be relocated by means of the vernier on the microscope stage. Their localisation was made possible by the use of a field finder, a microscope slide with a ruled, lettered microgrid. Taylor's finding ⁽¹⁰⁾ that cells subjected to repeated washings and soakings for immunofluorescence showed morphological deterioration was unfortunately amply confirmed. Staining was of an adequate quality to allow easy discrimination between polymorphonuclear and mononuclear cells, but only a few of the large mononuclear cells could /

could be categorised as monocytes or large lymphocytes.

For any one series of cell preparations, the titres obtained were consistent, and the same set of preparations was always used in the comparison of pre- and post-adsorptive titres of antiserum. It was noted, however, that higher titres of crude and thymocyte-adsorbed antiserum were obtained against mononuclear leucocytes than against leukaemic monocytes. Whilst it is conceivable that this could have been due to a lower density of antigen on the leukaemic cell than on normal monocytes, a more likely explanation is that the end-point of titration is strictly dependent on the density of the antigen-bearing cells in the preparation under study, consumption of antibody diminishing the fluorescence in cell-rich preparations. For the exact comparison of titres against different cell preparations, a method should be devised to prepare a standard carpet of cells on the microscope slide with which a standard volume of antiserum could be reacted within a circumscribed area.

Loewi et al ⁽²²⁾ were forced to abandon immunofluorescence in their research on anti-macrophage serum, because of the nonspecific fluorescence displayed by macrophages in suspension and in frozen tissue sections. This problem was almost completely circumvented by the use of fixed cell specimens, recommended by Nairn, ⁽⁹⁾ and by the application of low concentrations of fluorescent conjugate. Although nonspecific staining of neutrophil polymorphs, monocytes and macrophages was not entirely eliminated, it was found that adequate allowance for this background could be made in controls. Other workers have used immunofluorescence /

immunofluorescence successfully to detect anti-macrophage activity. (23)

RAMS was shown by immunoelectrophoresis to have a strong Ig M component; however the commercial FITC-conjugated goat-anti-rabbit immunoglobulin was raised against purified Ig G, and would bind to Ig M only by cross-reaction with light-chain determinants. A more sensitive detection of cell-bound Ig M antibodies from RAMS would have been possible, if FITC-conjugated anti-Ig M immunoglobulin had been used.

Cytotoxicity

The cytotoxicity test yielded reproducible results under standard conditions. The complement activity in the reaction mixture was of critical importance, and to standardise the dose employed in tests, the haemolytic potency of the serum source against sensitised SRBC was titrated, giving a measure of the complement activity in a parallel system, and the serum diluted accordingly. It was found that human serum was quite ineffective as a source of complement in the cytotoxic system of human cells and rabbit antiserum, but that guinea-pig serum had the necessary reactivity. Human serum had the potential disadvantage of the presence of isoantibodies against the cells under test; non-immune guinea-pig serum was shown to have no natural antibodies against human cells. Both human and guinea-pig sera have been reported to be weak or completely inert as sources of complement in cytotoxicity systems for the detection of human isoantibodies, (24) whereas rabbit serum has been widely applied in this capacity to great effect. However, the nature of rabbit serum's enhancement of antibody cytotoxicity has been shown to be as much supplementary as complementary, (25) being /

being due in part to the presence of low-affinity anti-human antibodies. Therefore, although it might have added to the sensitivity of the system, rabbit serum was not suitable for comparative titrations, where the activity of the serum used as a source of complement had to be standardised.

The cytotoxic titres of GPAMS derived by the method employed in this project were not as impressive as those obtained by some workers with animal anti-macrophage serum. Whereas Gallity ⁽²⁵⁾ used long incubation times of up to four hours, Engelfreit and Britten ⁽²⁷⁾ in a study of the variables involved in cytotoxicity tests, have shown that considerable nonspecific damage of cells occurs after 2 hours incubation, and it was considered prudent to restrict the incubation to 1 hour in the present study. The cinematographic recording of the cytotoxic effect of GPAMS demonstrated that immune destruction of cells can be very rapid.

Rosette inhibition

This technique proved a very sensitive device for revealing the presence of antibodies binding to the surface of receptor-bearing cells. It was capable of detecting antibody activity against the leukaemic monocyte in GPAMS from which all demonstrable cytotoxicity had been adsorbed. The antiserum was used at a standard dilution of 1 in 6 throughout, and at this concentration, antibodies remaining in the serum after adsorption with thymocytes and B lymphocytes inhibited the expression of the Ig G receptor on the leukaemic monocyte, but did not affect the complement receptor. A titration of the inhibitory power of GPAMS on each type of receptor before and after each adsorption would disclose whether inhibition of /

of the Ig G receptor was affected by adsorption. If the strong blockade of the Ig G receptor is due to its close proximity on the cell surface to a monocyte-specific antigen, it might be expected that the inhibitory titre would change very little after each adsorption.

B) General

The experimental results show that an antiserum has been raised against human leukaemic monocytes in the rabbit and in the guinea-pig.

Appropriate adsorption has led to the isolation of antibodies in GPAMS with a restricted specificity for 10 to 15% of human mononuclear leucocytes. Of these cells, a number could be identified as monocytes by their morphology, whereas others could only be designated as large mononuclear leucocytes. Adsorbed GPAMS also bound to splenic histiocytes.

Specificity of hetero-antisera

In recent years, a number of workers have succeeded in producing heterologous antisera with specificity for macrophages. The bulk of the work has centred on the mouse peritoneal exudate as the source of cells for inoculation, and the rabbit as the species in which to raise the antiserum. Early reports from Panijel and Cayeux (6, 28) indicated that antiserum raised in this way was cytotoxic to mouse macrophages, and had an immunosuppressive effect in vivo, but lacked absolute specificity, showing a degree of cross-reaction with lymphocytes.

Unanue (29) used a preparation of peritoneal exudate cells which had been cultured to reduce lymphocyte contamination, for inoculation, and reported /

reported that the resultant antiserum had very little anti-lymphocyte activity. Jennings and Hughes (30) described an anti-macrophage serum containing no lymphocyte agglutinins or cytotoxins, but more recent, fuller investigations of the specificity of rabbit anti-mouse-macrophage hetero-antibodies suggest that this claim was rather extravagant; antibodies cross-reacting with erythrocytes and lymphocytes are invariably produced, but can be removed by adsorption, leaving macrophage-specific activity in the serum. These findings are supported by accounts of cross-reactivity against macrophages in heterologous anti-lymphocyte serum in various species, which suggest the existence of antigens shared by the two types of cells.

The antigenic relationship between neutrophil polymorphs and macrophages has received only a short scrutiny. Gallily (26) demonstrated the presence of strong anti-neutrophil polymorph cytotoxicity in anti-macrophage serum in vitro, and Jasin et al (31) noticed a transient drop in the number of circulating neutrophil polymorphs after injection of mice with anti-macrophage serum which had been adsorbed with thymocytes.

The results from animal work parallel those of the present study of the human monocyte/macrophage family.

Source of cells for inoculation

No records of an attempt to raise hetero-antibodies against human macrophages or monocytes have yet appeared in the literature, and in fact, apart from lymphocytes, very few classes of human cells have been /

been used to produce antisera. In recent years, improved methods for the separation of cells from peripheral blood have extended the range of human cell types which could be obtained in populations pure enough for the immunisation of animals. Mahmoud and his colleagues have produced antisera which, after adsorption, could discriminate between human neutrophils, basophils, eosinophils, and myeloblasts. (32) The cells used in the production of anti-basophil, anti-eosinophil, and anti-myeloblast sera came from the blood of leukaemic patients.

The opportunity to undertake the present study was afforded by the withdrawal of blood from a patient suffering from a rare type of monocytic leukaemia. The detailed examination of the mononuclear cells separated from this blood in Dr Stuart's laboratory found them to resemble normal monocytes in their surface receptor characteristics, their phagocytic capacity, and their ability to mature into macrophages. Enumeration of the cells having receptors for SRBC, and those bearing surface immunoglobulin, indicated that T and B lymphocytes, respectively, accounted for less than 5 per cent of the population. Thus the starting inoculum for the raising of antiserum was a highly pure suspension of apparently normal human monocytes. It was assumed at the outset that the monocyte and the macrophage would be at least closely related antigenically, if not identical, and that an anti-monocyte serum would have anti-macrophage activity. This premise was not without foundation, as Feldman et al (33) have described a macrophage-specific surface antigen in the guinea-pig, present in a high concentration on peritoneal macrophages, and in a lower density on peripheral monocytes. The brilliant staining of splenic histiocytes by GPMS which had been adsorbed /

adsorbed with thymocytes and erythrocytes suggests a similar relationship between the cytoplasmic antigens of human monocytes and macrophages.

Significance of adsorption procedures

Adsorption with the cells used for inoculation is an important check in any study of a hetero-antiserum; an infection in the animal, concurrent with the immunisation injection series can give rise to anti-microbial antibodies cross-reacting with human cell antigens. GPAMS was rendered immunologically impotent by adsorption with monocytes.

The antibodies in untreated RAMS and GPAMS were active against surface and cytoplasmic antigens of a spectrum of human cell types, and it is highly likely that some of these were species antigens common to all human cells. When all haemagglutinating activity had been removed from RAMS by adsorption with erythrocytes, reactivity against leucocyte cytoplasmic antigens was undiminished, indicating a qualitative antigenic difference between the two classes of blood cells.

Adsorption of the antiserum with human thymocytes was calculated to remove anti-lymphocyte and anti-human antibodies. The resultant antiserum, no longer active against thymocytes or against platelets, had no evident cytotoxicity against leukaemic monocytes, but at low dilutions killed a fraction of cells in a mononuclear leucocyte suspension which could be accounted for by the number of monocytes plus contaminating neutrophil polymorphs. This finding in no way casts suspicion on the validity of the cytotoxic test, rather, it illustrates the resistance of tumour cells to immunological attack, whether by means of a reduced density of surface antigens /

antigens, or a thicker hide. The thymocyte-adsorbed GPAMS marked out a subclass of the mononuclear cells of human peripheral blood in immunofluorescent tests. Some of these cells were unequivocally monocytes, and removal of thymocyte-reactive antibodies clearly left an antiserum of very restricted specificity. The existence of distinct antigenic determinants on T and B lymphocytes has been recognised in the mouse, and recent evidence suggests a similar situation in man. It was argued that a further adsorption with B cells would eliminate any anti-B-lymphocyte antibodies which had escaped the thymocyte net. However, following this second adsorption, the percentage of mononuclear cells binding GPAMS was unchanged. It must be concluded, therefore, that the doubly-adsorbed GPAMS had a specificity, which excluded conventionally defined T and B lymphocytes, for human monocytes, and possibly a small number of related lymphocytes. Fuller investigation of the identity of this defined group of cells is required, and could be approached by enzyme studies.

Throughout all of the adsorption procedures, GPAMS faithfully retained its anti-neutrophil polymorph activity, and the nature of the antigenic relationship between monocyte and polymorph merits further inquiry by adsorption.

The possibility that the antiserum might contain antibodies against tumour-specific antigens borne by the leukaemic monocytes as well as those directed against normal monocyte antigens, was not investigated, but the fact that adsorbed GPAMS retained activity against leukaemic monocytes leaves this possibility open.

Evidence for surface activity of GPAMS

The results of receptor studies on cells treated with GPAMS clearly point to an interaction between antibodies and cell surface components, causing interference with the ability of cell surface receptors to bind their complementary molecular structures.

The inhibition, by anti-lymphocyte serum, of Ig G and complement receptors on human monocytes has been used by Huber et al to detect its anti-monocyte activity. (13) In the present study, this property of surface-reacting antibodies was used to monitor the activity of GPAMS over the course of successive adsorptions, and showed that antibodies directed against the leukaemic monocyte cell membrane remained after adsorption with human thymocytes and CLL cells. The observation that unadsorbed GPAMS, shown to be active against thymocytes by cytotoxicity and immunofluorescence, failed to inhibit formation of SRBC rosettes on these cells, was unexpected. Bach and Dormont (34) have found anti-human-thymocyte sera to inhibit SRBC rosetting on peripheral blood lymphocytes, although they have not found this inhibiting capacity to correlate with their cytotoxic potency.

When it was found that a fraction of peripheral blood leucocytes was killed by thymus-adsorbed antiserum, the experiment of incubating these cells with adsorbed GPAMS and complement, and then testing their receptor function, was performed in the belief that a subclass of non-T cells were being killed, and T cells spared.

The /



The observation of complete inhibition of surface receptor expression on mononuclear leucocytes by thymocyte-adsorbed GPAMS is difficult to reconcile with the uninhibited thymocyte rosetting and the specificity of the adsorbed antiserum in immunofluorescence and cytotoxicity tests. Harmony is restored if it is accepted that thymocytes and T lymphocytes are antigenically different, as is suggested by Ishii et al, and that rosette inhibition is a much more sensitive technique for the detection of antibody binding than is immunofluorescence and cytotoxicity.

It has been shown that the Ig G receptor and the complement receptor are inhibited by anti-immunoglobulin antibodies, and this might explain the extraordinary finding of Huber et al ⁽¹³⁾ that anti-lymphocyte serum adsorbed with lymphocytes continued to block Ig G and complement receptors. GPAMS was shown in double diffusion tests to be free from activity against human serum proteins.

The potentiation of rosette inhibition by complement, reported by Reyes and Bach ⁽²⁵⁾ and refuted by Brain et al ⁽³⁶⁾, was confirmed. The mechanism of this potentiation is obscure; it is certainly not dependant on cell killing as recognised by the uptake of trypan blue, but could be due to a sublethal cytopathic effect causing an alteration in the cell membrane properties. Alternatively, or in addition, a steric inhibition of membrane receptors by surface-bound antibodies could be enhanced more passively by the attachment of the large molecules of the complement sequence.

The different susceptibilities of the complement and the Ig G receptors to /

to inhibition by surface-bound antibodies previously demonstrated in the mouse macrophage by Holland et al (37) and in the human monocyte by Huber et al (13) was also noted here. The latter group have shown that inhibition of complement receptors by anti-lymphocyte serum titrates out before Ig G receptor inhibition. These findings suggest that the two receptors are located differently in relation to monocyte surface antigens, and leave open the possibility that antibodies could be directed against Ig G receptor itself. In the present study, the finding that Ig G receptor inhibition remained after adsorption with B lymphocytes which possessed surface Ig G receptors suggests that it was not the receptor itself but something situated close to it, that was the subject of the surface activity of GPAMS.

Further evidence for interaction with the cell plasma membrane was afforded by time-lapse cinematographic recording of the effect of the antiserum on the behaviour of leukaemic monocytes. These cells, cultured on glass coverslips, made particularly interesting subjects for visual study, by virtue of their motility, and spreading forms. The rapid withdrawal of pseudopodia caused by low dilutions of GPAMS indicated that the binding of antibody and complement to the plasma membrane first evokes an active response from the cell. The ensuing changes in morphology were consistent with an irreversible breakdown in membrane integrity under enzymatic attack, leading to the influx of water and disruption of organelles, the accepted sequence of events in complement-mediated cell lysis.

PROJECTIONS

Now that the practicability of raising heteroantiserum against the human monocyte has been demonstrated, the production of more antiserum is called for. Antiserum with a degree of specificity similar to that of the adsorbed GPAMS in this study could be applied to an immunofluorescent investigation of the nature of the Reed-Sternberg cell of Hodgkin's disease, and of the mesangial cell of the human glomerulus.

Further work is required to discover the mechanism of receptor blockade by antibodies binding to the cell surface, and to explain the different effects of GPAMS on thymocyte and peripheral blood T lymphocyte receptors for SRBC.

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